Effect of Bcl-2 on the cellular response to oxidative stress

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Abstract

Exposure of cells to hydrogen peroxide (H_2O_2) can cause oxidative damage to cellular constituents including lipids, protein, and DNA. At elevated concentrations, H_2O_2 can trigger cell death by apoptosis or necrosis. Apoptotic cell death can be prevented by overexpression of the oncoprotein Bcl-2. The exact mechanism by which Bcl-2 blocks cell death is controversial. Some researchers believe that Bcl-2 possesses antioxidant properties that protect cells from apoptosis. The purpose of this thesis was to assess oxidative stress and apoptosis following H_2O_2 exposure in Jurkat T cells overexpressing Bcl-2. One of the major objectives was to ascertain whether or not Bcl-2 overexpression elevated the antioxidant capacity of Jurkat T cells to provide protection from oxidant-induced cell death.

H_2O_2 treated Jurkat cells became apoptotic at moderate levels of oxidant (25-100 μM H_2O_2), and necrotic at higher doses (>200 μM H_2O_2). Bcl-2 overexpression prevented caspase activation and cell death at the apoptotic doses of H_2O_2, but not the necrotic doses. Caspase inhibition studies demonstrated that Bcl-2 overexpression provided a greater level of resistance from H_2O_2-induced cell death than the broad-spectrum caspase inhibitor z-VAD.fmk.

A systematic study was carried out examining the antioxidant status of Jurkat cells overexpressing Bcl-2. Several Bcl-2 transfectants were utilised for the study, so that any differences seen could be correlated to the level of Bcl-2 expression. Surprisingly, there were no statistically significant differences among the Bcl-2 transfectants for any of the antioxidant enzymes.

Jurkat cells overexpressing Bcl-2 exhibited the same level of oxidative damage to lipids and protein in response to H_2O_2 exposure as the parental Jurkat cells. Interestingly, Jurkat cells overexpressing Bcl-2 continued to grow in culture after H_2O_2 exposure, despite harboring damage to cellular constituents. Consistent with

these results, H_2O_2 treated Jurkat cells overexpressing Bcl-2, which failed to undergo apoptosis, were more prone to genomic instability.

Together, these findings suggest that Bcl-2 overexpression protects Jurkat cells from H_2O_2-induced cell death by blocking apoptosis. Jurkat cells overexpressing Bcl-2 were no better at detoxifying oxidants and showed the same level of oxidative damage following H_2O_2 exposure. As a result, the overexpression of Bcl-2 considerably enhanced the mutagenicity of H_2O_2.

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Abbreviations

8-oxo-Dg	8-hydroxy-deoxyguanosine
AIF	apoptosis inducing factor
AMC	7-amino-4-methylcoumarin
ANOVA	analysis of variance
ANT	adenine nucleotide transporter
Apaf-1	apoptotic protease activating factor
ARE	antioxidant responsive element
ATM	ataxia telangiectasia mutated protein
Bak	Bcl-2-antagonist/killer
Bax	Bcl-2-associated X protein
Bid	BH3 interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-interacting mediator
Bmf	Bcl-2 modifying factor
Bok	Bcl-2-related ovarian killer
Bcl-2	B cell lymphoma-2
Bcl-x_L	Bcl-2-like 1 isoform
Bcl-w	Bcl-2-like 2 protein
BH	Bcl-2 homology
BSA	bovine serum albumin
CBMN	cytokinesis block micronuclei assay
CED-9	cell death protein 9
CHAPS	3-[3-chloramidopropyldimethylammonio]-1-propane-sulphonate
c-Myc	c-myelocytomatosis
CN	cyanide
Cyp-D	cyclophilin D
cyt c	cytochrome c
dATP	deoxyribose adenine triphosphate
DC	detergent compatible
DEVD-AMC	Asp-Glu-Val-Asp-7-amino-4-methylcoumarin

DIABLO	direct IAP-binding protein with low pI
DMSO	dimethylsulphoxide
DNA	deoxyribosenucleic acid
DNP	2,4-dinitrophenylhydrazine
DSB	double strand breaks
DTNB	5,5’-dithiobis(2-nitrobenzoic acid)
DTPA	diethylenetriaminepentaacetic acid
DTT	dithiothreitol
EBV	Epstein-Bar virus
ECL	electrochemiluminescence
EDTA	ethylenediaminetetraacetic acid
Egl-1	egg-laying defective protein 1
EGTA	ethylene glycol (2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Em	emission wavelength
Ex	excitation wavelength
FACS	fluorescent activated cell sorter
FBS	fetal bovine serum
Fe^{2+}	iron
FITC	flourescein-isothiocyanate
FKBP38	FK-506 binding protein homologue 38
FOX	ferrous oxidation of xylenol orange
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gpx	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulfide
Grx	glutaredoxin
hMYH	mutY homolog
hOGG1	8-oxoguanine DNA glycosylase isoform 1
H_2O	water
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hrk	harakiri
HRP	horseradish peroxidase

HtrA	high temperature requirement protein A
IAF	5-iodoacetamidoflourescein
IAP	inhibitor of apoptosis protein
IC_{50}	50% inhibitory concentration
Ig	immunoglobulin
Keap1	Kelch-like ECH-associated protein 1
KSV	Kaposi sarcoma virus
LD_{50}	50% lethal dose
LH	lipid
$LOO^{\cdot}$	lipid peroxyl radical
LOOH	lipid hydroperoxide
NAC	N-acetyl cysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NMR	nuclear magnetic resonance
$NO^{\cdot}$	nitric oxide
Nrf2	nuclear transcription factor erythroid 2p45-related factor 2
Mcl-1	myeloid cell leukemia sequence 1
MDA	malondialdehyde
MDR	multi-drug resistance
MN	micronuclei
MOMP	mitochondrial outer membrane permeabilisation
MPT	mitochondrial permeability transition
NF-κB	nuclear factor kappa light polypeptide gene enhancer in B-cells
$O_2^{-\cdot}$	superoxide
$OH^{-\cdot}$	hydroxyl radical
$ONOO^{-\cdot}$	peroxynitrite
p53	phosphoprotein of 53 kDa
PBS	phosphate buffered saline
PI	propidium iodide
PTP	permeability transition pore
Prx	peroxiredoxin
Puma	p53 up-regulated modulator of apoptosis

PVDF	polyvinylidene difluoride
Rac1	ras-related C3 botulinum toxin substrate 1
RAD51	recA-like gene cDNA 51
$R^{\cdot}$	radical
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RSH	free thiol
RSOH	sulfenic acid
RSO_2H	sulfinic acid
Se	selenium
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Smac	second mitochondria-derived activator of caspase
SOD	superoxide dismutase
TBS-T_{20}	Tris buffered saline containing Tween^{TM} 20
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethylthylenediamine
TNB	5-thio-2-nitrobenzoic acid
Tris	tris-(hydroxymethyl)-methylamine
Trx	thioredoxin
TrxRed	thioredoxin reductase
WST-1	water soluble tetrazolium dye
z-VAD.fmk	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethylketone

Chapter 1: Introduction

The oxidant H_2O_2 can damage cellular constituents and trigger the programmed cell death pathway apoptosis. Apoptosis can be blocked by overexpression of the oncoprotein Bcl-2. Many investigators believe that Bcl-2 also possesses antioxidant properties that protect cells from oxidative stress. The purpose of this thesis was to examine the consequences of H_2O_2-mediated oxidative stress in Jurkat T lymphocytes overexpressing Bcl-2, and to determine if antioxidant properties of Bcl-2 contribute to the protection that Bcl-2 provides from oxidative stress.

1.1 Oxidative stress

Oxidative stress arises from a cellular imbalance between oxidants and antioxidants culminating in oxidant generation which exceeds the detoxification capacity of the cell (Finkel and Holbrook 2000). Reactive oxygen species (ROS), produced endogenously or derived from external sources, pose a threat to most major cellular substituents including lipids, protein and DNA (Figure 1.1). Oxidative damage can inhibit proliferation (growth-arrest), cause premature ageing (cellular senescence), and in some circumstances trigger cell death (Chandra et al. 2000; Finkel 2003). Aerobic cells have evolved to cope with the toxicity of ROS by utilizing a range of enzymatic and non-enzymatic antioxidants (Ames et al. 1993; Halliwell 1996). These antioxidants function by either scavenging or repairing damage caused by ROS.

The two major ROS produced endogenously are superoxide ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2). Although there are many endogenous sources of ROS, by far the majority (~90%) is produced by the mitochondrial electron transport chain during oxidative phosphorylation (Balaban et al. 2005). $O_2^{-\cdot}$, formed via the incomplete reduction of molecular oxygen during mitochondrial respiration, dismutates spontaneously and via an enzyme-catalysed reaction to form H_2O_2. While $O_2^{-\cdot}$ and H_2O_2 may cause damage in some circumstances, their effects are more dramatic in combination with other ROS (Beckman and Koppenol 1996; Halliwell 1996). For

instance, $O_2^{-\cdot}$ can combine with endogenous nitric oxide ($NO^{\cdot}$) to form peroxynitrite ($ONOO^{-\cdot}$), a reactive nitrating agent (Beckman and Koppenol 1996). Similarly, H_2O_2 can participate in Fenton chemistry by reacting with free iron (Fe^{2+}) to produce the hydroxyl radical ($OH^{-\cdot}$), a very reactive and indiscriminant oxidant (Wardman and Candeias 1996).

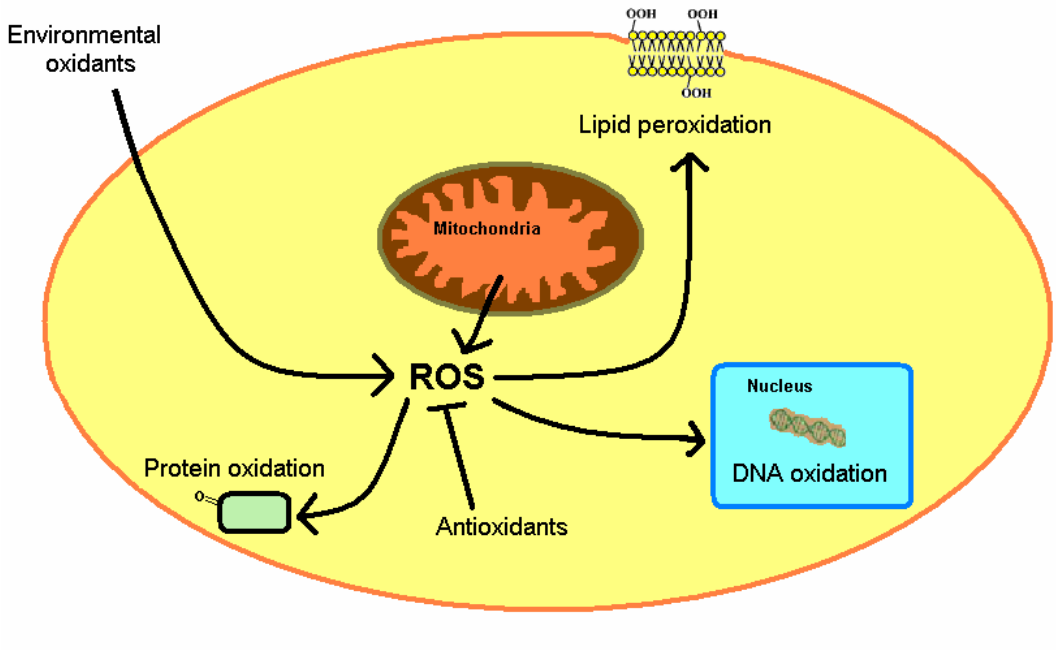


Figure 1.1: ROS can cause oxidative damage to cellular components (Finkel and Holbrook 2000).

1.1.1 Consequences of oxidative stress in cultured cells

Lipid peroxidation

Lipids are particularly susceptible to oxidation due to their abundance at sites of ROS generation (Niki et al. 2005). Lipid peroxidation is a process initiated by the abstraction of a hydrogen atom from a fatty acid chain (LH) by a radical ($R^{\cdot}$). The resulting carbon centred lipid radical reacts immediately with oxygen to form the peroxyl radical ($LOO^{\cdot}$), which can propagate lipid peroxidation by abstracting a hydrogen atom from neighboring lipids (Porter et al. 1995).

1. Initiation $LH + R^{\cdot} \rightarrow L^{\cdot} + RH$
2. Propagation $L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$
3. Propagation $LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$

The chain reaction results in the accumulation of lipid hydroperoxides (LOOH), which perturb membrane structure and participate in further redox reactions. This includes the formation of toxic aldehyde products such as malondialdehyde (MDA) that cause further damage to the cell by modifying proteins (Esterbauer et al. 1991).

Oxidation of proteins

The oxidation of proteins can severely compromise cellular function by inactivating enzymes, compromising structural proteins and disrupting protein-protein interactions involved in signal transduction pathways (Martinez-Sanchez et al. 2005). One of the most widely studied products of protein oxidation are protein carbonyls (Berlett and Stadtman 1997). Protein carbonyls can be formed by the oxidation of lysine, arginine, proline, and threonine residues. In addition, carbonyl moieties can be introduced into proteins by electrophilic modification, with aldehydes formed during lipid peroxidation (Figure 1.2) (Esterbauer et al. 1991).

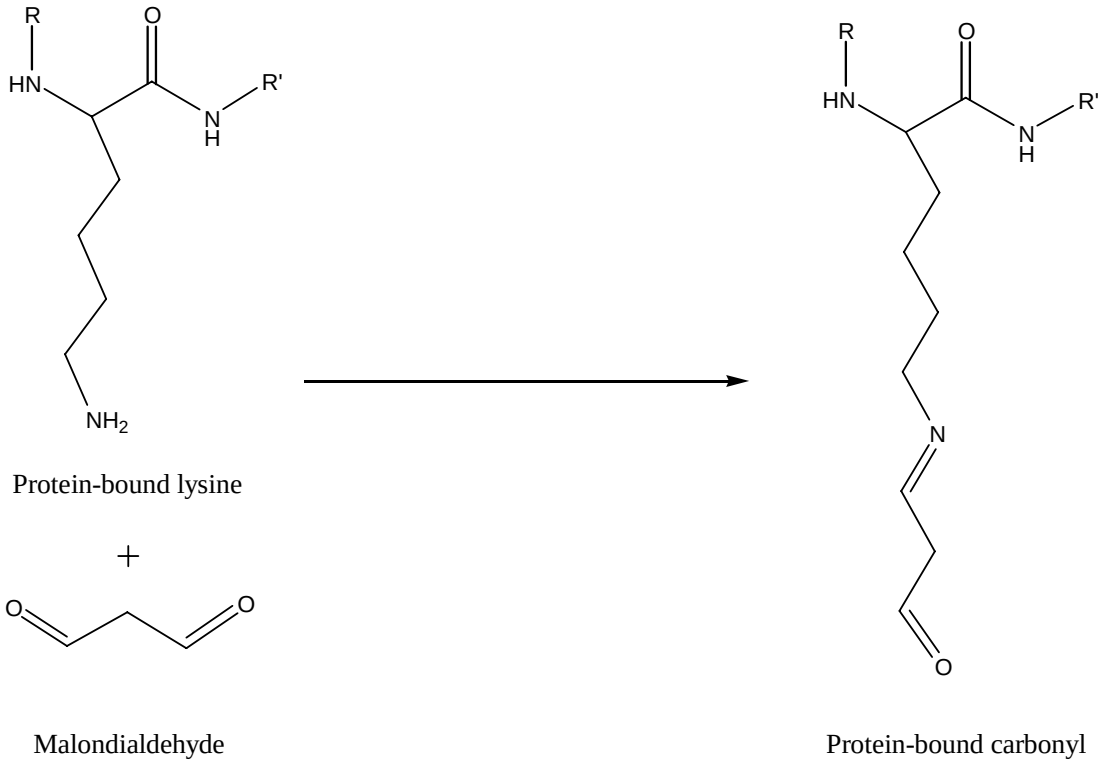


Figure 1.2: Example of protein carbonyl formation. *Reactive aldehydes such as malondialdehyde can react with basic residues to form protein carbonyl derivatives (Esterbauer et al. 1991).*

Cysteine residues (Cys-SH) within proteins are highly sensitive to changes in the redox status of the cell (Finkel 2000; Ghezzi 2005). H_2O_2 can oxidise cysteine residues within proteins to form sulfenic acids (Cys-SOH), sulfinic acids (Cys-SO_2H), intra- and inter-disulfides or mixed disulfides with glutathione (Jacob and Sies 2003) (Figure 1.3).

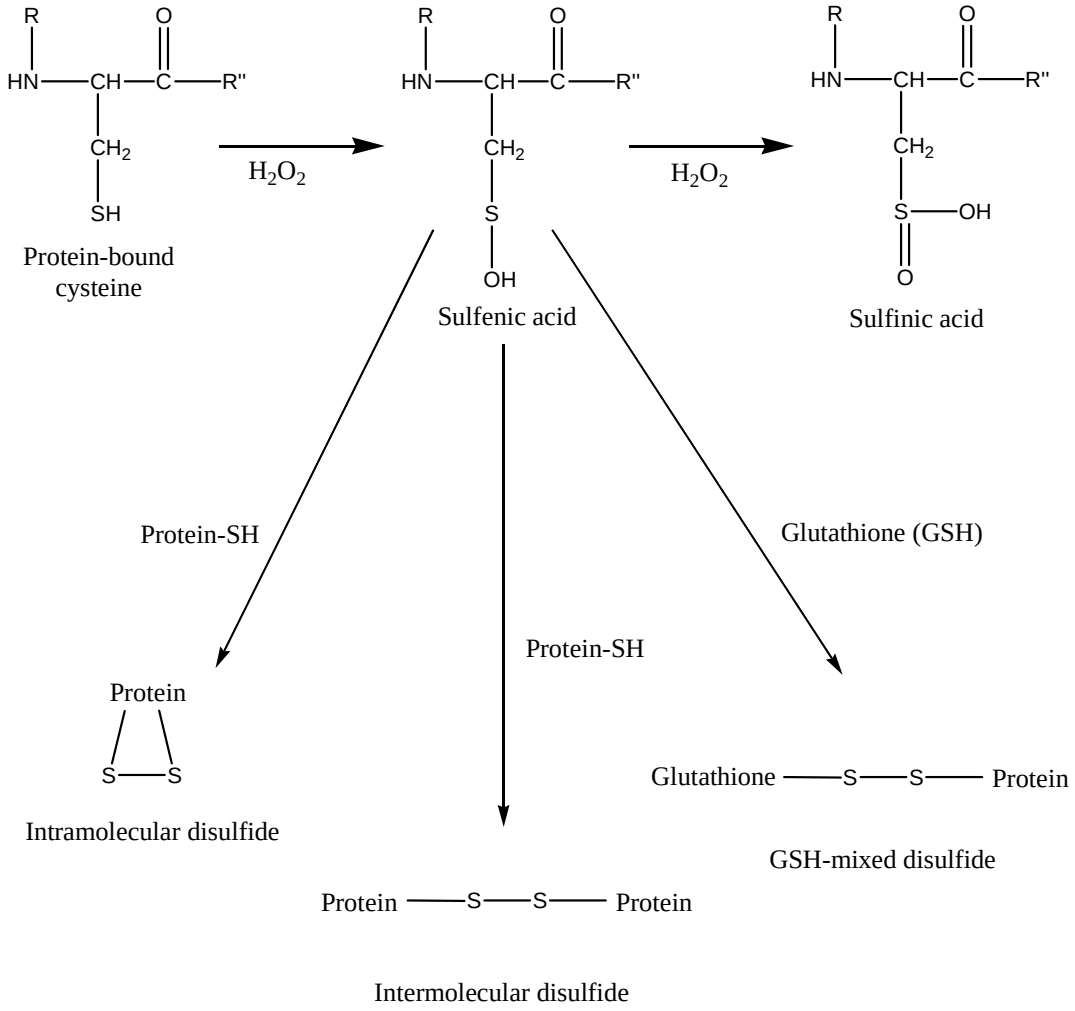


Figure 1.3: Protein thiol oxidation products*. ROS such as H_2O_2 react with protein thiols to form a variety of oxidised thiol derivatives (Ghezzi 2005).*

It has become increasingly apparent that cells are able to use the oxidation of reactive cysteine residues to sense changes in ROS levels in their immediate environment (Cross and Templeton 2004; Forman et al. 2004). This phenomenon was first characterised in bacteria, where a cysteine residue in the OxyR transcription factor is oxidised, leading to activation and increased transcription of antioxidant and repair

enzymes (Zheng et al. 1998; Choi et al. 2001; Lee et al. 2004). A variety of other redox-sensitive thiol proteins have been discovered in eukaryotes (Li and Karin 1999; Delaunay et al. 2000; Dinkova-Kostova et al. 2002; Wood et al. 2003a), although their importance in the cellular responses to oxidative stress is unclear. The factors responsible for the greatly increased reactivity of some cysteines also remain to be completely characterised, but one important factor is the presence of positively charged residues in the immediate vicinity that lower the pKa of the cysteine sulfhydryl (Wood et al. 2003a; Forman et al. 2004).

DNA damage

ROS can severely damage DNA to form oxidised derivatives such as 8-hydroxy-deoxyguanosine (8-oxo-dG) (Halliwell and Aruoma 1991; Valko et al. 2004) (Figure 1.4). Under normal circumstances, these defects are repaired by the DNA base excision repair pathway. However, in cells exposed to chronic oxidative stress, 8-oxo-dG accumulates. 8-oxo-dG codes for adenosine instead of cytosine, initiating a deleterious G→T transversion mutation (Klein et al. 1992).

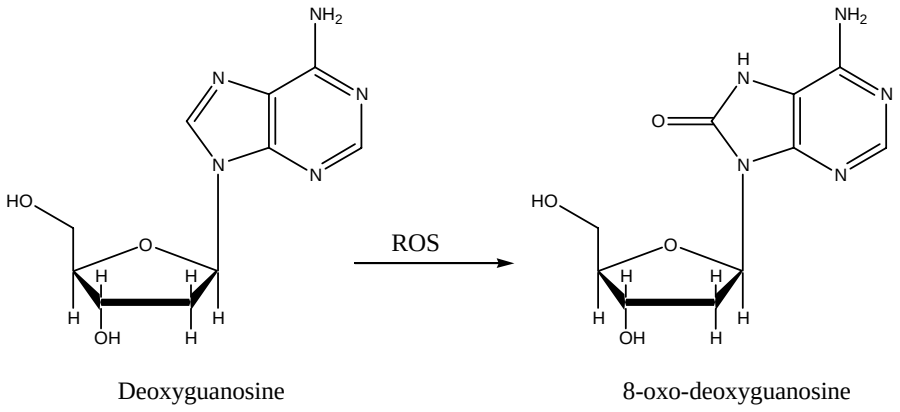


Figure 1.4: DNA base oxidation. *ROS oxidise DNA to form 8-oxo-deoxyguanosine (Halliwell and Aruoma 1991).*

In addition to modifying nucleosides, ROS can attack the DNA backbone, forming DNA double strand breaks (DSBs) (Jackson et al. 1998). Left unaided, DSBs trigger chromosomal translocations and promote persistent genomic instability, a process characterized by the formation of chromosome aberrations that are transmissible to daughter cells (Zhivotovsky and Kroemer 2004).

1.1.2 Cellular responses to oxidative stress

The cellular response to ROS is largely dependent on the level of oxidant exposure. Low levels of H_2O_2 activate gene expression to induce proliferation, presumably by acting as a second messenger molecule in mitogenic signaling pathways (Burdon et al. 1996; Finkel 2000). Elevated levels of oxidant can activate a number of stress pathways, including temporary growth arrest, cellular senescence, apoptosis and necrosis (Davies 1999).

Cells exposed to a moderate level of oxidative stress can become temporarily growth-arrested (Davies 1999). Cells downregulate the transcription of housekeeping genes and activate the transcription of stress-responsive genes designed to repair oxidative damage (Wiese et al. 1995; Crawford et al. 1996; Li and Karin 1999). Hence, temporary growth arrest is an adaptive response that allows cells to cope with moderate oxidant exposure.

Cellular senescence is a stress-responsive program activated in response to a high level of oxidative stress, and is designed to block the replication of damaged cells (Sherr and DePinho 2000). ROS can trigger cellular senescence in a p53-dependent pathway mediated by DNA damage (Ben-Porath and Weinberg 2005). Senescent cell populations disregard mitogenic signals and become permanently growth arrested. In the growth-arrested state, morphological and metabolic changes occur that parallel changes seen in aging cells (Campisi 1996; Sherr and DePinho 2000).

If oxidative stress results in extensive irreparable damage to the cell, a cell death program called apoptosis is activated (Green 1998; Finkel 2003). Apoptotic cells exhibit common morphological changes including cell shrinkage, nuclear condensation and membrane blebbing (Kerr et al. 1972). These morphological characteristics are associated with the activation of intracellular proteases known as caspases (Thornberry and Lazebnik 1998). Apoptosis is an energetic process requiring ATP, and can be activated and modulated by perturbations in the redox tone of the cell, as many factors involved are highly redox-sensitive (Hampton and Orrenius 1998; Cross and Templeton 2004). Cells exposed to extremely high or sustained levels of ROS undergo a caspase-independent form of cell death known as necrosis (Chandra et al. 2000; Zong and Thompson 2006). The necrotic pathway is

characterized by a loss of membrane integrity that causes the release of noxious oxidants, which promote inflammatory responses in surrounding tissue. Apoptosis cannot occur under extreme oxidative stress because there is a loss of cellular ATP (Eguchi et al. 1997) and the caspases contain an active site cysteine residue that is susceptible to oxidative inactivation (Hampton and Orrenius 1997; Hampton et al. 2002).

1.1.3 Involvement of ROS in carcinogenesis and tumour development

There is mounting evidence that ROS play a major role in the initiation of diseases such as cancer (Behrend et al. 2003; Storz 2005). The incidence of cancer rises sharply with age, suggesting that age-related defects at the cellular level might be responsible for increased susceptibility to this disease (Finkel 2005). It is generally accepted that mitochondrial respiratory function declines with ageing, leading to an increase in ROS production, which in turn, promotes further damage (Balaban et al. 2005). Many believe that the "vicious cycle" of ROS production, caused by mitochondrial dysfunction, is a primary factor involved in the age-related increase in susceptibility to cancer. In support of this theory, mice with impaired antioxidant defence, have an increased rate of spontaneous tumour formation (Neumann et al. 2003). Furthermore, many studies have documented that antioxidant supplementation can prevent the formation of tumours in cancer-prone mice (Schubert et al. 2004; Sablina et al. 2005), reinforcing the view that ROS drive tumour progression (Sablina et al. 2005). Enhanced ROS production can promote tumourigenesis by providing a mutagenic environment that facilitates genomic instability (Vafa et al. 2002; Sablina et al. 2005). In addition, ROS can promote malignant transformation towards a metastatic phenotype (Radisky et al. 2005). Consistent with the role of ROS in tumour development, established cancer cells have been shown to produce elevated levels of ROS, including H_2O_2 (Szatrowski and Nathan 1991).

1.1.4 Cellular defence against oxidative stress

Direct antioxidant scavengers

Antioxidants that directly scavenge ROS form the first line of defence against oxidative stress. Some antioxidant scavengers, including vitamin C and E, are not synthesized by humans and therefore need to be obtained from the diet. Vitamin C (ascorbate) scavenges ROS by donating an electron to form the relatively unreactive

semidehydroascorbate radical, which is regenerated to ascorbate and dehydroascorbate via a disproportionation reaction (Kojo 2004). Vitamin E (α-tocopherol) is a hydrophobic antioxidant that protects the cellular membrane from lipid peroxidation by scavenging lipid peroxyl radicals (Singh et al. 2005). The resultant α-tocopherol radical can be recycled to its original form by vitamin C (Halliwell 1996).

Superoxide dismutase (SOD) is an enzymatic antioxidant that forms the cornerstone for defence against ROS (Fridovich 1995). As the name implies, its function is to catalyse the dismutation of $O_2^{-\cdot}$ to H_2O_2. The two major isoforms of SOD (CuZn-SOD and Mn-SOD) reside in distinct cellular compartments. CuZn-SOD (SOD1) is a 32 kDa cytosolic protein that relies on zinc to stabilise the enzyme and the redox chemistry of copper (Cu^{2+}) to catalyse superoxide dismutation. Mn-SOD (SOD2) is a 40 kDa tetrameric protein that resides exclusively in the mitochondrial matrix (MacMillan-Crow and Cruthirds 2001). Although Mn-SOD is less abundant than CuZn-SOD, its importance is demonstrated by the finding that Mn-SOD deficient mice die within 2 weeks of birth (Li et al. 1995).

One of the predominant enzymes involved with decomposing H_2O_2 is catalase. Catalase is a heme-based protein that decomposes two molecules of H_2O_2 to form water and oxygen (Aebi 1984). The first molecule of H_2O_2 reacts with the heme in catalase to form compound 1, which can then react with a second molecule of H_2O_2 to regenerate the active form of catalase.

Thiol-dependent antioxidants

Most proteins rely on cofactors such as transition metals and flavins to catalyse redox chemistry. In contrast, cysteine and selenocysteine residues can endow proteins with the capacity to participate in a variety of redox reactions (Jacob and Sies 2003). The reactivity of cysteine and selenocysteine is exploited in numerous antioxidants including glutathione, thioredoxin, glutaredoxin, thioredoxin reductase, glutathione peroxidase, and the peroxiredoxins.

Glutathione (GSH) is a tripeptide (γ-glutamyl-cysteinyl-glycine) that plays a major role in maintaining the redox balance of the cell (Sies 1999). It is the most abundant non-protein thiol in the cell by several orders of magnitude. GSH can scavenge ROS directly or act as a co-factor for enzymatic defenses. The oxidised form of glutathione can be regenerated in a NADPH-dependent manner by glutathione reductase. Thioredoxin (Trx) is a low molecular weight protein with a conserved Cys-X-X-Cys motif that catalyses the reduction of protein disulfides (Burke-Gaffney et al. 2005) (Figure 1.5). Thiol exchange reactions performed by Trx result in the formation of an intramolecular disulfide, which can be resolved by thioredoxin reductase. The related protein, glutaredoxin (Grx), utilises the reducing power of GSH to catalyse disulfide reduction (Fernandes and Holmgren 2004) (Figure 1.6A). In mammalian cells, there are two isoforms of Trx and Grx, one of which is cytosolic, whilst the other resides in mitochondria (Vlamis-Gardikas and Holmgren 2002).

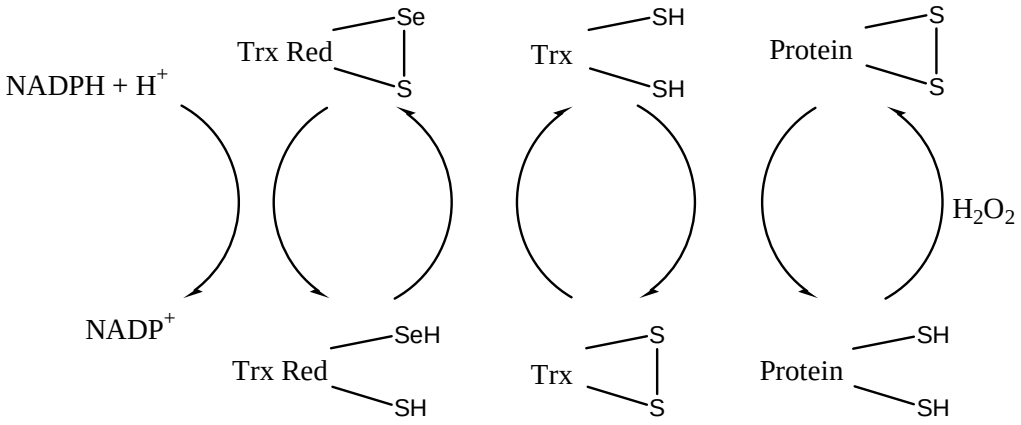


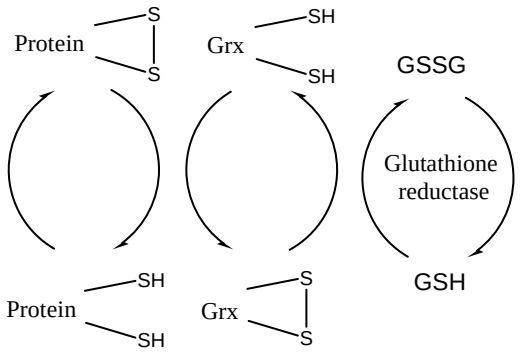
Figure 1.5: Thiol exchange reactions of the thioredoxin pathway. *Thiol transfer reactions involving thioredoxin and thioredoxin reductase drive the reduction of oxidised thiols (Holmgren et al. 2005).*

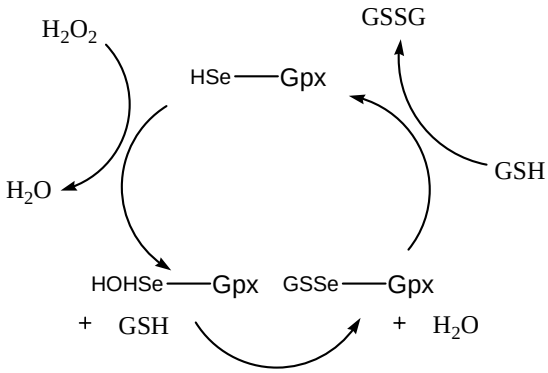
Thioredoxin reductase (TrxRed) is a selenoprotein that regenerates oxidised Trx at the expense of NADPH (Arner and Holmgren 2000). There are several TrxRed isoforms (1-3) that provide complementary activity in the cytosol (TrxRed 1 and 3) and mitochondria (TrxRed 2). The Trx/TrxRed system provides significant protection from ROS by maintaining a thiol rich intracellular environment. The selenocysteine, present in the active site, provides a nucleophilic moiety ideal for the enzymatic reduction of Trx. Interestingly, the selenocysteine is exposed at the carboxyl terminus

of the protein, which enables it to reduce substrates other than Trx such as Grx and vitamin C (May 2002).

Glutathione peroxidase (GPx) is a ubiquitous antioxidant enzyme that uses two molecules of GSH to reduce H_2O_2 to H_2O (Flohe and Gunzler 1984; Lei 2002) (Figure 1.6B). GPx has a selenocysteine in the active site that promotes the nucleophilic decomposition of H_2O_2. Several isoforms of GPx (GPx 1-6) exist that differ in their structure and localisation. Gpx 1 resides in the cytoplasm and mitochondria, while Gpx 3 is secreted into the circulation. GPx 4 is a unique isoform that specifically reduces alkyl hydroperoxides such as lipid hydroperoxides (Imai and Nakagawa 2003; Ran et al. 2005).

A)





B)

Figure 1.6: Glutathione cellular reduction pathways. *Glutathione participates in redox reactions with Grx to reduce protein thiols (A), and Gpx to detoxify ROS (B) (Sies 1999).*

The peroxiredoxins (Prxs) decompose H_2O_2 by exploiting the reactivity of a conserved cysteine residue termed the peroxidatic cysteine (Cys-S_pH) (Rhee et al. 2005a). The active site of Prx contains positively charged residues that lower the pKa of the peroxidatic cysteine, stabilising the thiolate anion (Cys-S_p^-). The various isoforms of Prx exist in specific subcellular locations such as the cytosol (Prx 1,2 and 6), the mitochondria (Prx 3), the extracellular space (Prx 4) and the peroxisome (Prx 5) (Wood et al. 2003b). The Prxs are one of the most highly abundant proteins in the cell (around 0.1-0.8% of the total protein), which compensates for their moderate

catalytic efficiency (Rhee et al. 2005c). During the catalytic cycle, the oxidised peroxidatic cysteine forms a disulfide bond that can be resolved by Trx (Figure 1.7) (Nakamura 2005). The Prxs become inactivated in response to high doses of H_2O_2, due to the overoxidation of the peroxidatic thiol (Cys-S_pH) to a sulfinic acid (Cys-S_pO_2H) (Georgiou and Masip 2003). However, the overoxidised form of peroxiredoxin can be slowly reduced in an ATP-dependent pathway involving the enzyme sulfiredoxin (Biteau et al. 2003). In addition to its role as an antioxidant, Prx regulates H_2O_2-mediated signaling pathways in the cell (Rhee et al. 2005b). Overexpression of Prx enzymes can reduce H_2O_2 generated in response to cytokine receptor activation and modulate signaling downstream of receptor stimulation (Zhang et al. 1997; Kang et al. 1998; Kang et al. 2004; Rhee et al. 2005b). For example, the mitochondrial isoform of Prx (Prx 3) can modulate apoptotic signal transduction by altering the levels of H_2O_2 present in the mitochondria (Chang et al. 2004).

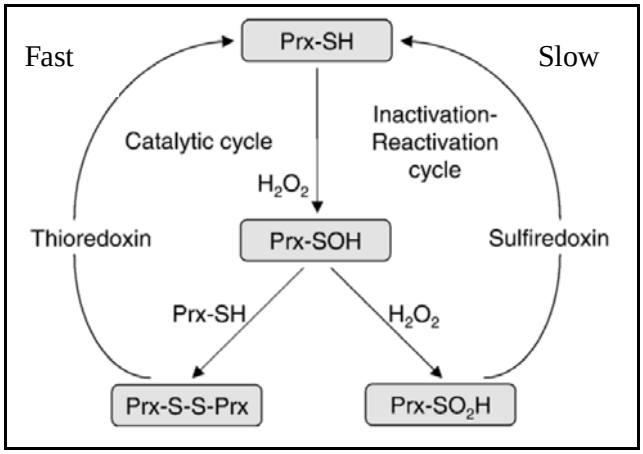


Figure 1.7: Catalytic cycle of the peroxiredoxins *(figure adapted from Rhee et al. 2005a).*

Indirect antioxidants

The term antioxidant has evolved to include entities that can indirectly influence the antioxidant capacity of the cell. For instance, the trace mineral selenium (Se) is viewed as an antioxidant, in light of its integral function as a redox catalyst in selenoproteins, such as Gpx and TrxRed (Tapiero et al. 2003). The expression of selenoproteins is compromised by a limited selenium supply (McKenzie et al. 2002). Hence, selenium can modulate the expression of essential antioxidant defense systems. Similarly, metal binding proteins such as albumin, ferritin, transferrin,

ceruplasmin, and metallothioneins are viewed as antioxidants by virtue of their ability to sequester redox-active transition metals and thereby prevent ROS formation caused by Fenton chemistry (Halliwell 1996; Wardman and Candeias 1996).

Electrophilic thiol-modifying agents, such as polyphenols and the isothiocyanates, form a novel class of antioxidant that function by activating the expression of endogenous antioxidant defenses, termed the phase 2 response (Talalay et al. 2003; Keum et al. 2004; Masella et al. 2005). Paradoxically, inducers of the phase 2 response act by oxidizing or alkylating cellular thiols. The dosage is key; at lower doses, such compounds trigger the phase 2 adaptive response, but at higher doses these compounds can be potent inducers of apoptosis (Thomson et al. 2006). The phase 2 response is mediated by the transcription factor Nrf2 (Chen and Kong 2004). When active, Nrf2 translocates to the nucleus where it binds to conserved ARE (antioxidant responsive element) sequences in the promoter region of phase 2 genes to activate transcription. Under basal conditions, Nrf2 is sequestered in the cytosol by Keap1, which is anchored to the actin cytoskeleton. Electrophilic compounds trigger the phase 2 response by directly modifying highly reactive cysteine residues in Keap1 (Dinkova-Kostova et al. 2002). Cysteine modifications in Keap1 cause a conformational change to occur that releases Nrf2, allowing it to translocate to the nucleus and activate the phase 2 response (Itoh et al. 1999).

Other proteins, such as c-Myc, p53, Rac1, ATM and Bcl-2 have recently been proposed to indirectly alter the redox status of the cell (Hockenbery et al. 1993; Polyak et al. 1997; Vafa et al. 2002; Reliene et al. 2004; Schubert et al. 2004; Singh et al. 2004). In each case, the mechanism by which these proteins alter cellular redox is controversial. The antioxidant properties of Bcl-2 are the focus of this thesis.

1.2 Bcl-2

Research on Bcl-2 has increased exponentially since its discovery more than twenty years ago. The sustained research effort is due to both the realisation of its central role in the regulation of apoptosis, and the association of Bcl-2 with diseases such as cancer. Bcl-2 was first identified as a proto-oncogene present at the breakpoint of a

chromosomal rearrangement in B cell follicular lymphoma (Pegoraro et al. 1984; Tsujimoto et al. 1984; Bakhshi et al. 1985). A cytogenetic analysis of the lymphoma cells revealed a distinctive translocation of chromosomes 14 and 18. The t(14;18) chromosome rearrangement repositioned the Bcl-2 gene from its normal position on chromosome 18 into the vicinity of an immunoglobulin heavy chain intron enhancer present on chromosome 14. The resulting translocation caused a transcriptional activation of the Bcl-2 gene, and, therefore, an increase in the abundance of the oncoprotein (Graninger et al. 1987; Reed et al. 1987; Seto et al. 1988).

Bcl-2 is the founding member of a family of homologous proteins whose interactions dictate the fate of a cell (Adams and Cory 1998). The Bcl-2 family is broadly categorized into two groups that regulate apoptosis (figure 1.8). The anti-apoptotic members of the Bcl-2 family such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and CED-9 promote cell survival. The pro-apoptotic members (Bax, Bak, Bok, Bik, Hrk, Bim, Noxa, Bad, Puma, Bmf, EGL-1 and Bid) are activated by apoptotic stimuli and promote cell death (Willis and Adams 2005; Liu et al. 2006). The ratio of the two antagonising groups determines whether a cell resists or succumbs to an apoptotic stimulus (Korsmeyer et al. 1993; Korsmeyer 1999). The high level of redundancy in the family is due to the tissue and stimulus specific expression of various Bcl-2 family members. Sequence homology between Bcl-2 family members is confined to Bcl-2 homology (BH) domains, which are known to be important for protein-protein interactions between the family members (Hanada et al. 1995).

Since the discovery of Bcl-2, homologues have been found in lower eukaryotes such as nematodes (*Caenorhabditis elegens*) (Hengartner and Horvitz 1994), fruit flies (*Drosphila melanogaster*) (Quinn et al. 2003), and mosquitoes (*Anopheles gambiae*) (Igaki and Miura 2004), illustrating the likelihood that Bcl-2 function is conserved throughout metazoan evolution. Bcl-2 homologues are present in both Epstein-Barr virus (EBV) (Henderson et al. 1993) and Kaposi's sarcoma virus (KSV) (Cheng et al. 1997; Polster et al. 2004). These animal viruses have been implicated in cancer and other disorders. It is likely that EBV and KSV have usurped ancestral forms of the Bcl-2 gene from past hosts to exert a survival advantage in infected cells (Parris 2005).

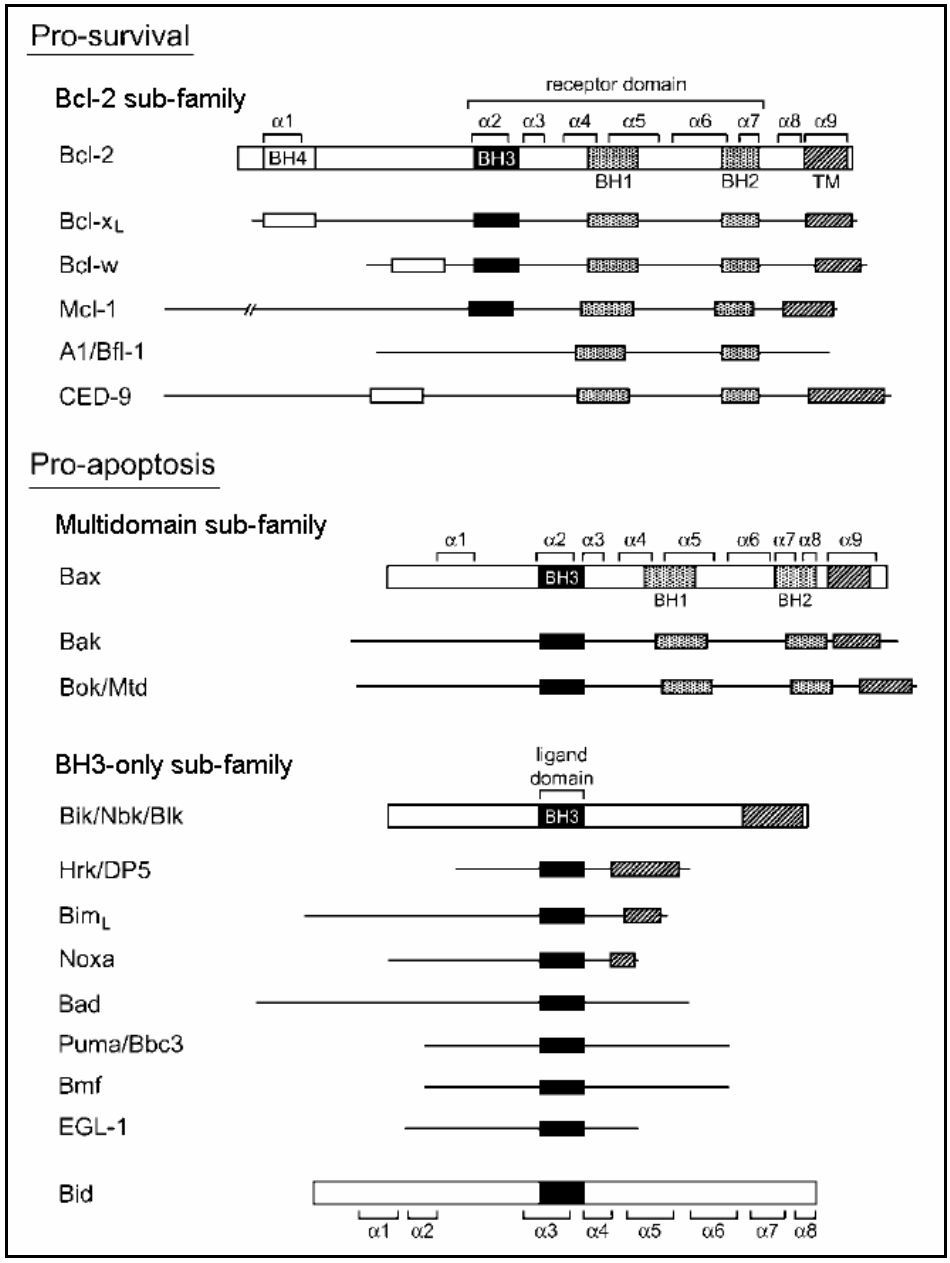


Figure 1.8: The Bcl-2 family of proteins *(figure from Adams and Cory 1998).*

1.2.1 Role of Bcl-2 in tumourigenesis and cancer

There has been a long association between Bcl-2 and tumourigenesis. Tumourigenesis is a multi-step process involving the progressive transformation of normal cells into neoplastic clones via the acquisition of essential traits. At several stages in the transformation process, cancer cells can be exposed to a wide variety of apoptosis-inducing stimuli, such as genotoxicity, uncontrolled proliferation, oxygen and nutrient deprivation, and the loss of cell adhesion (Kirkin et al. 2004). Consequently, one on the six essential traits of a cancerous cell is the ability to evade apoptosis (Hanahan

and Weinberg 2000). Evasion of apoptosis can be fulfilled by the overexpression of anti-apoptotic proteins such as Bcl-2. Thus, Bcl-2 represents a unique type of proto-oncogene that promotes tumourigenesis by preventing apoptosis.

Investigations with transgenic mouse models have provided tantilising evidence for the tumourigenic potential of Bcl-2. Pioneering studies by Reed et al. demonstrated that when nude mice were injected with Bcl-2 transfected fibroblasts, they developed tumours with transformed morphology (Reed et al. 1988). Similar studies were performed using Bcl-2-Ig fusion transgenic mice that mimicked the chromosome translocation present in follicular lymphoma (McDonnell et al. 1989; McDonnell and Korsmeyer 1991). The Bcl-2-Ig transgenic mice displayed an increase in the survival of B cells, but otherwise no ill effects(McDonnell et al. 1989). However, after a long latency period, the B cells become malignant and the transgenic mice develop diffuse large B-cell lymphomas (McDonnell and Korsmeyer 1991). The phenotype of the Bcl-2-Ig transgenic mice reflects the stages of follicular lymphoma, in which non-threatening lymphadenopathy can exist for years before transforming to a more aggressive form of the disease. A recent report using conditional Bcl-2/Eu-Myc transgenic mice has verified that apoptotic evasion is essential for the maintenance of leukemia (Letai et al. 2004). The transgenic mice used in the study developed lymphoblastic leukemia with pronounced genomic instability. However, when Bcl-2 was downregulated in the transgenic mice, the leukemic cells became apoptotic, leading to tumour remission and a significantly prolonged survival. Collectively, these data suggest that tumourigenic cells constantly convey death signals that are blocked by Bcl-2 overexpression (Letai 2005b; Letai et al. 2005).

The overexpression of Bcl-2 has been associated with many forms of cancer including B cell lymphoma (Tsujimoto et al. 1985), acute lymphoblastic leukemia (CoustanSmith et al. 1996), gastric cancer (Ayhan et al. 1994), colorectal carcinoma (Ayhan et al. 1994), and prostate cancer (Krajewska et al. 1996). The overexpression of Bcl-2 in such cancers can render tumour cells resistant to radiotherapy and chemotherapeutic drugs (Green and Kroemer 2005; Reed and Pellecchia 2005). Hence, Bcl-2 has been likened to multidrug resistance (MDR) proteins (Reed 1999). However, unlike the MDR efflux pumps, Bcl-2 does not protect cells from the cytotoxic effects of a drug, it just prevents the resulting cell death from occurring.

Naturally, given its oncogenic potential, Bcl-2 has been a target for drug discovery (Juin et al. 2004), the assertion being that inhibition of Bcl-2 would remove the anti-apoptotic status that cancer cells had exploited to resist chemotherapy. To date, a range of small molecule inhibitors of Bcl-2 have been shown to sensitise drug-resistant cancer cells to apoptosis (Wang et al. 2000; Enyedy et al. 2001; Juin et al. 2004; O'Neill et al. 2004; Letai 2005a; Oltersdorf et al. 2005; Reed and Pellecchia 2005). The most successful of these, gossypol, is currently in phase 2 clinical trials (Pellecchia and Reed 2004; Reed and Pellecchia 2005).

Another approach used to sensitise cancer cells to apoptosis involves inhibiting Bcl-2 gene expression with nuclease resistant antisense RNA therapy (Green and Kroemer 2005; Letai 2005b; Reed and Pellecchia 2005). Bcl-2 antisense therapy has been shown to enhance apoptosis alone, and in combination with, conventional chemotherapeutics in numerous xenograft mouse models of solid tumours and haematological malignancies (Cotter et al. 1994; Jansen et al. 1998; Klasa et al. 2000; Leung et al. 2001; Hu et al. 2004; Smith et al. 2004; De Cesare et al. 2005). The Bcl-2 antisense therapeutic, Oblimersen (G3139, Genasense), has had encouraging results in the clinical setting, and is currently in phase 3 clinical trials in combination with conventional chemotherapeutics for the treatment of chronic lymphocytic leukemia, multiple myeloma, non-Hodgkin’s lymphoma and non-small cell lung carcinoma (Chanan-Khan 2004; O'Neill et al. 2004; Piro 2004; Rudin et al. 2004; Chanan-Khan 2005; Tolcher 2005).

1.2.2 Structure of Bcl-2

Primary structure: Importance for subcellular localisation

Bcl-2 has four BH domains (denoted BH1, BH2, BH3 and BH4) each of which is vital for function (Hanada et al. 1995). All members of the Bcl-2 family share the BH3 domain; however, the BH4 domain is only present in the anti-apoptotic members of the family (Huang et al. 1998). The primary structure of Bcl-2 reveals the presence of a hydrophobic stretch of 19 amino acids at the C-terminus. The hydrophobic C-terminal domain is thought to form an α-helix that can span the lipid bilayer, thereby acting as a transmembrane anchor to target Bcl-2 to internal membranes within the cell. Studies have revealed that the majority of Bcl-2 is localised to the mitochondrial

outer membrane (Hockenbery et al. 1990; Nguyen et al. 1993); however, a large proportion is also present at the endoplasmic reticulum and nuclear envelope (Krajewski et al. 1993; Lithgow et al. 1994). Recent studies have shown that Bcl-2 is specifically targeted to the mitochondrial outer membrane, via interactions with membrane bound scaffolding protein FKBP38 (Shirane and Nakayama 2003). Deletion of FKBP38 leads to a redistribution of Bcl-2 and a marked increase in apoptosis.

Tertiary structure of Bcl-2

Structural data on Bcl-2 have been notoriously difficult to obtain due to the insolubility of the hydrophobic C-terminal domain and the unstructured nature of the loop domain (Petros et al. 2001). Nevertheless, these problems were eventually resolved and the solution structure of Bcl-2 was elucidated by NMR spectroscopy (Petros et al. 2001) (Figure 1.9). The tertiary structure of Bcl-2 reveals a predominantly α-helical protein containing eight α-helices. The two central hydrophobic α-helices (α5 and α6) are surrounded by four amphipathic α-helices forming a distinctive hydrophobic groove. The BH1-BH3 domains of Bcl-2 form the hydrophobic groove whilst the BH4 domain stabilises the entire structure by burying hydrophobic residues (Yan and Shi 2005). Site-directed mutagenesis of the BH1 and BH2 domains inactivate Bcl-2 (Yin et al. 1994), demonstrating that the hydrophobic groove is necessary for Bcl-2 function. A long loop domain, which is devoid of secondary structure, exists between α1 and α2 and functions as a regulatory domain (Chang et al. 1997).

A conserved Asp-Trp-Gly-Arg (NWGR) sequence in the BH1 domain is important for both the structural integrity and function of Bcl-2 (Petros et al. 2004). Studies have demonstrated that deletion of this conserved NWGR motif leads to the inactivation of Bcl-2 (Hanada et al. 1995). The tryptophan residue (W) in the NWGR sequence makes extensive hydrophobic interactions with α7 and α8 locking the BH1 and BH2 domains together to define the top of the hydrophobic groove. The arginine residue (R) in the NWGR sequence provides a positive charge at the top of the hydrophobic groove that is essential for the heterodimerisation function of Bcl-2 (Hanada et al. 1995).

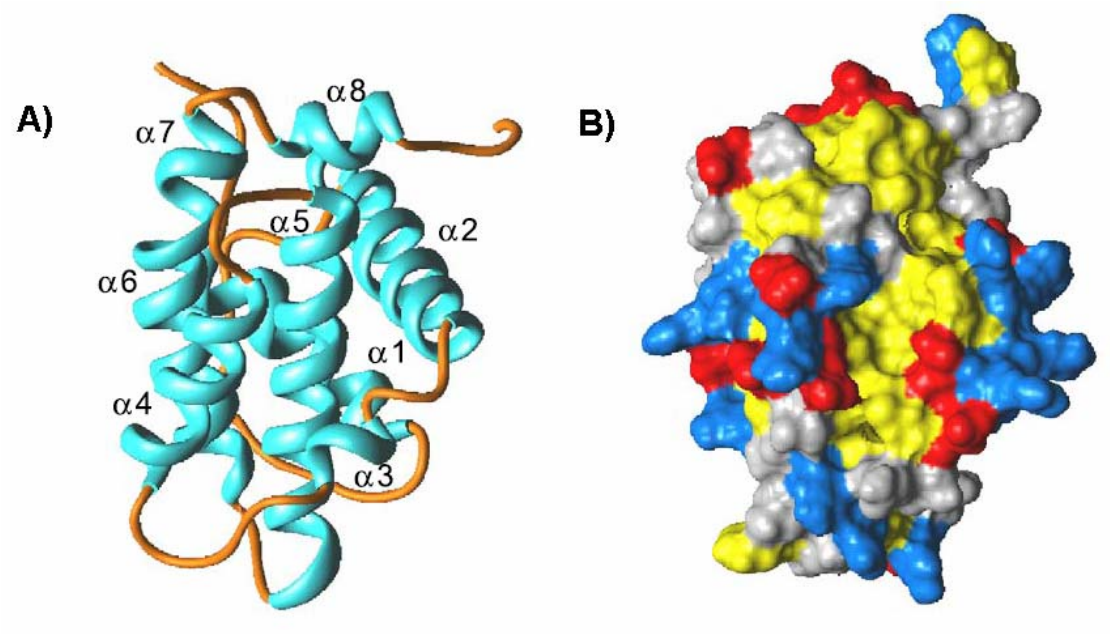


Figure 1.9: Ribbon representation (A) and Connelly surface structure (B) of Bcl-2. *Yellow coloured residues are hydrophobic, while red and blue coloured residues are acidic and basic respectively (Figure from Petros et al. 2004).*

Structural insights into Bcl-2 function: Pore formation

Bcl-2 family structures show a surprising homology with bacterial pore-forming toxins such as diptheria and colicin. This homology inspired several studies that examined the pore-forming ability of Bcl-2 family members (Schendel et al. 1997). By analogy to the bacterial toxins, the investigators proposed that the two hydrophobic α-helices (α5 and α6) could span the membrane, while the amphipathic α-helices assembled above the lipid bilayer like an umbrella. A functional pore with an aqueous lumen could, therefore, be envisaged, consisting of dimers or oligomers of Bcl-2. Biophysical studies by Schendel et al. (Schendel et al. 1997) have provided proof of principle using recombinant Bcl-2 in planar lipid bilayers to form a functional pore. However, pore formation required a pH lower than 4, an abundance of anionic lipids in the membrane and a trans-negative membrane potential. Such conditions would be difficult to emulate *in vivo*; therefore, the functional significance of pore formation by Bcl-2 is not known.

Structural insights into Bcl-2 function: Heterodimerisation

The predominant function of Bcl-2 is to heterodimerise with pro-apoptotic members of the Bcl-2 family. The structural requirements for heterodimerisation have been clarified with NMR-derived structural studies on the related protein Bcl-x_L (Sattler et al. 1997). The solution structure of a Bcl-x_L/Bak complex revealed the importance of the hydrophobic groove in recognising pro-apoptotic members of the Bcl-2 family at the molecular level. The structural data verified that neither the BH1 or BH2 domains of pro-apoptotic family members are necessary for heterodimerisation (Chittenden et al. 1995). Instead, Bcl-x_L interacted specifically with the BH3 domain of Bak. The BH3 domain adopts an amphipathic α-helix upon binding via both hydrophobic and electrostatic interactions. Conserved hydrophobic residues (Val74, Leu78, Ile81and Ile85) present on the BH3 peptide, align within the hydrophobic groove, while charged residues (Arg76, Asp83, and Asp84) form electrostatic bridges with oppositely charged residues within Bcl-x_L (Glu129, Arg139, and Arg100). The need for both hydrophobic and electrostatic interactions at the interface was confirmed by alanine scan mutagenesis of the BH3 peptide (Sattler et al. 1997). Peptide binding studies with Bcl-2 and BH3 peptides have shown similar hydrophobic and electrostatic requirements for efficient heterodimerisation (Petros et al. 2001) (Figure 1.10).

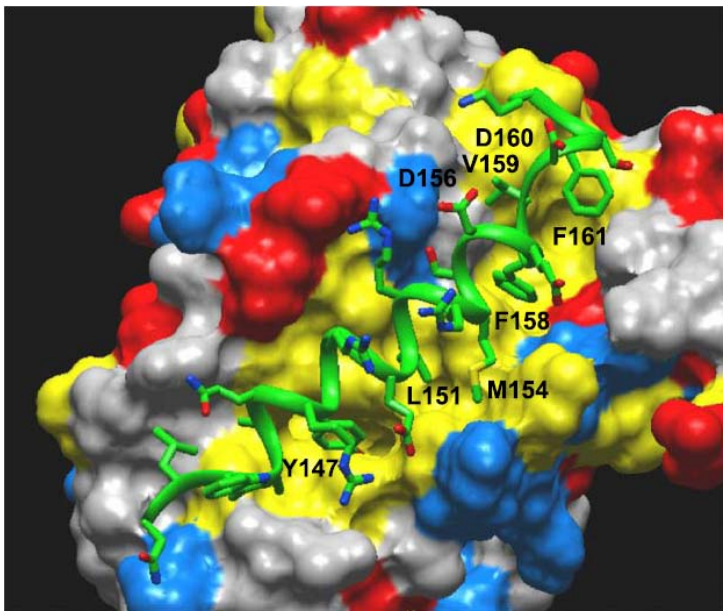


Figure 1.10: Heterodimerisation function of Bcl-2. *Bcl-2 heterodimerises to the "BH3" domain of pro-apoptotic members of the Bcl-2 family (figure from (Petros et al. 2004)).*

1.2.3 Apoptosis and its regulation by Bcl-2

Pathways of apoptosis

The two principle modes of apoptosis are termed the extrinsic and intrinsic pathways. The extrinsic pathway is mediated by death receptors including Fas (Nagata 1997). On ligand binding, Fas receptors oligomerise and recruit cytosolic adapter proteins to form the death-inducing signaling complex (DISC), which can activate caspase 8 and promote apoptosis (Kischkel et al. 1995; Muzio et al. 1996; Danial and Korsmeyer 2004). In contrast, the intrinsic or mitochondrial pathway is controlled by members of the Bcl-2 family. Multiple signals, including oxidative stress, activate the mitochondrial pathway making it the dominant form of programmed cell death (Green and Kroemer 2004) (Figure 1.11).

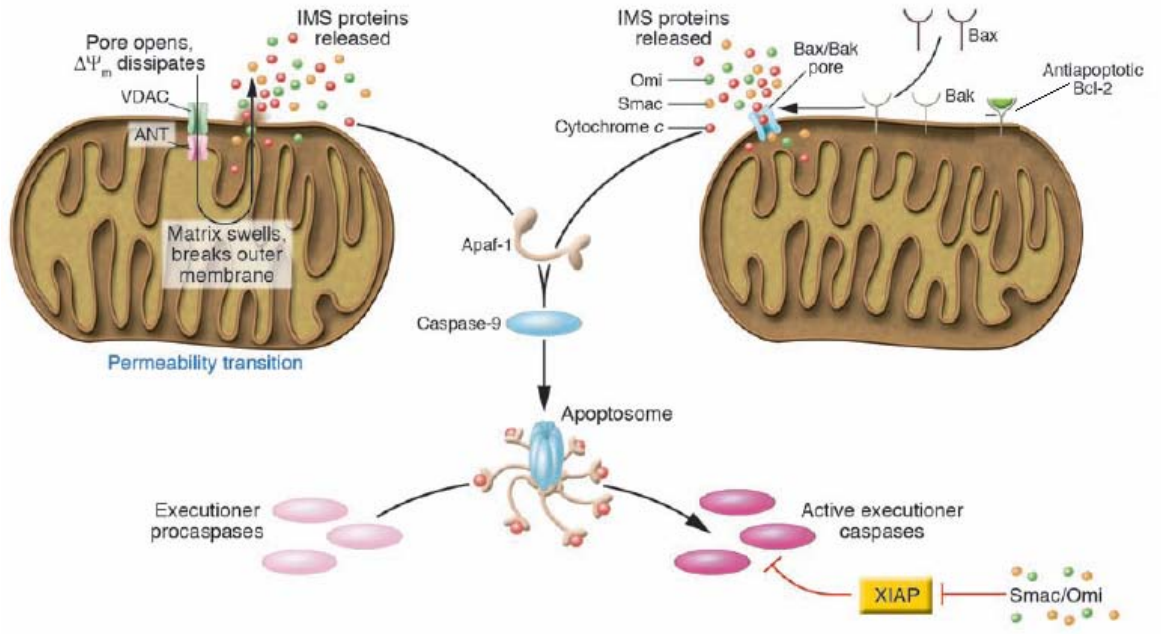


Figure 1.11: The mitochondrial pathway of apoptosis *(figure adapted from Green and Kroemer 2005).*

The mitochondrial pathway of apoptosis

During mitochondrial-mediated apoptosis, pro-apoptotic members of the Bcl-2 family become activated and promote mitochondrial outer membrane permeabilisation (MOMP). The permeabilisation of the mitochondria releases cytochrome c (cyt c), which forms a complex with Apaf-1 and dATP (Liu et al. 1996; Li et al. 1997). The cyt c/Apaf-1/dATP complex facilitates a conformational change in Apaf-1, exposing a

binding site for the “activator” caspase, procaspase-9. The resulting holoenzyme complex, termed the apoptosome, catalyses the auto-cleavage of procaspase 9 into the active form (Acehan et al. 2002). Caspase 9, in turn, cleaves and activates the “executioner” caspases (caspase 3 and 7). Executioner caspases cleave key proteins in the cell at conserved Asp-Glu-Val-Asp (DEVD) sequence motifs (Thornberry and Lazebnik 1998). The activation of the executioner caspases generally marks a point of no return in the apoptotic pathway (Cohen 1997; Salvesen and Dixit 1997).

In addition to cytochrome c, other pro-apoptotic factors such as AIF, Smac/DIABLO, endonuclease G and Omi/HtrA are released from mitochondria (Susin et al. 1999; Du et al. 2000; Verhagen et al. 2000; van Loo et al. 2001; van Loo et al. 2002b). Smac/DIABLO and Omi/HtrA act by binding, via their amino-termini, to endogenous IAPs (inhibitors of apoptosis proteins) (Vaux and Silke 2003). In healthy cells, IAPs sequester active caspases, thereby helping to prevent accidental cell death. By inhibiting IAP function, Smac/DIABLO and Omi/HtrA promote caspase activation (Chai et al. 2000; Verhagen et al. 2002). AIF and endonuclease G promote cell death by degrading DNA in a caspase-independent manner (Susin et al. 1999; van Loo et al. 2001). These apoptogenic factors act synergistically with cytochrome c to promote cell death (van Loo et al. 2002a).

Role of BH3-only proteins in apoptosis

Pro-apoptotic Bcl-2 family members that promote this sequence of events can be classified into two groups, the BH3-only proteins (Bid, Bim, Puma, Bad, Bmf, Bik, Hrk, and Noxa) and the multidomain proteins (Bax and Bak). BH3-only proteins are a structurally diverse group of proteins that become activated in response to death stimuli (Willis and Adams 2005) (Figure 1.12). Several BH3-only proteins are activated at the transcriptional level by growth factor deprivation (Bim and Hrk) and DNA damage (Noxa and Puma) (Oda et al. 2000; Nakano and Vousden 2001; Puthalakath and Strasser 2002; Willis and Adams 2005). The remainder of the BH3-only proteins are activated at the post-translational level. For example, Bmf and Bim are activated by detachment from the cytoskeleton in response to anoikis and UV radiation (Puthalakath et al. 1999). Bad activation requires dephosphorylation and dissociation from the adaptor protein 14-3-3 (Zha et al. 1996). Bid is cleaved by caspase 8 in response to death receptor activation (Li et al. 1998). The cleaved form,

tBid (truncated Bid), is then myristoylated, facilitating its relocation to the mitochondrial membrane (Zha et al. 2000). Bid cleavage allows cross talk between the extrinsic and intrinsic pathways of apoptosis. BH3-only proteins act as either "activators" or "sensitisers" (Letai et al. 2002; Kuwana et al. 2005). Activator BH3-only proteins (Bid, Bim and Puma) bind and activate multidomain pro-apoptotic members of the Bcl-2 family. In contrast, sensitiser BH3-only proteins (Bad, Bmf, Bik, Hrk, and Noxa) work by antagonizing anti-apoptotic members of the Bcl-2 family such as Bcl-2.

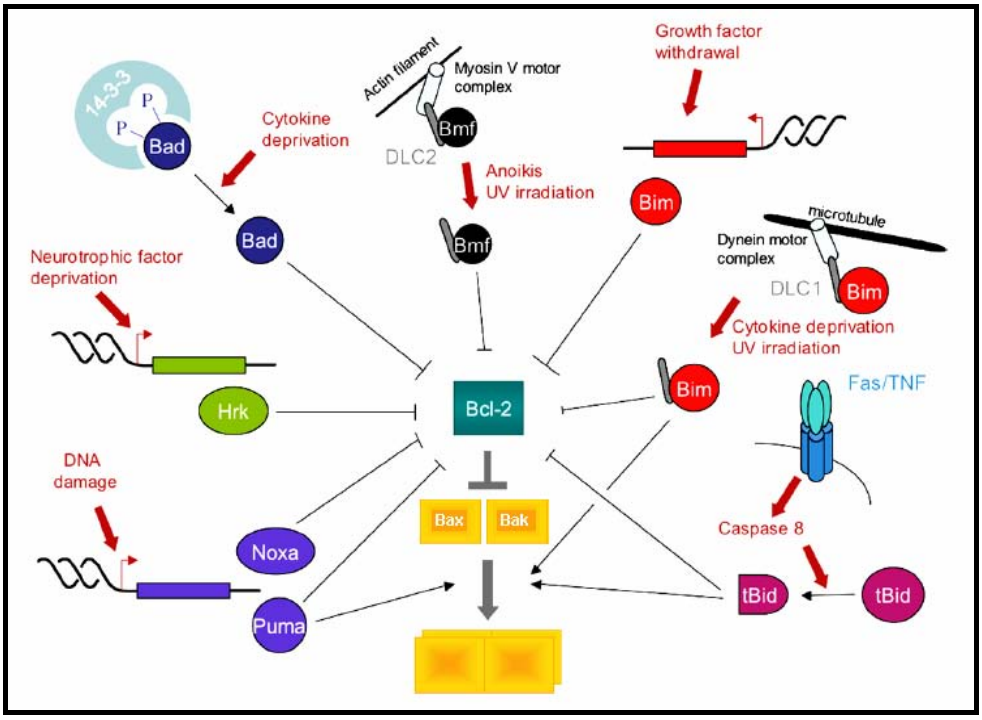


Figure 1.12: Death-inducing stimuli activate "BH3-only" proteins to promote apoptosis (figure adapted from (Willis and Adams 2005)).

Role of multidomain proteins in promoting apoptosis

The multidomain proteins, Bax and Bak, depend on activated BH3-only proteins to promote MOMP. The mechanism by which Bax and Bak function at the molecular level is debatable, however there are three major theories. The first theory suggests that Bax and Bak can alter the composition of the membrane allowing the formation of lipid pores (Basanez et al. 1999). In this model BH3-only molecules activate Bax, which promotes a positive membrane curvature and pore formation (Terrones et al. 2004).

The second theory proposes that the Bcl-2 family members regulate a pre-existing mitochondrial permeability transition pore (PTP) (Brenner et al. 2000). The PTP complex is composed of several mitochondrial proteins including VDAC, ANT, and cyclophilin D. Together these proteins form a pore that spans both the inner and outer mitochondrial membranes. The opening of such a pore leads to a phenomenon termed mitochondrial permeability transition (MPT) (Sharpe et al. 2004). The MPT process is characterized by an influx of protons, resulting in mitochondrial swelling and eventual membrane rupture. In support of this theory, Bax and Bak have been shown to bind to both VDAC and ANT, activating the PTP and triggering MPT (Marzo et al. 1998; Shimizu et al. 1999). However, recent studies using cyclophilin D knockout mice ($CypD^{-/-}$) have helped to clarify the importance of the PTP in cell death (Baines et al. 2005; Halestrap 2005; Nakagawa et al. 2005). Cyclophilin D deficiency prevents the formation of the PTP and, therefore, blocks the MPT. $CypD^{-/-}$ fibroblasts are more resistant to necrotic triggers of cell death, such as oxidative stress. However, $CypD^{-/-}$ and wildtype fibroblasts displayed similar sensitivities to apoptotic triggers. The emergent view is that the PTP regulates necrosis, but is not required for apoptotic cell death.

The third and most widely accepted theory is that, upon activation, multidomain pro-apoptotic proteins form de novo pores that cause MOMP (Green and Kroemer 2004). The formation of such pores requires the presence of activator BH3-only proteins, in order to facilitate translocation of Bax and Bak, to the mitochondria. Once at the mitochondria, conformational changes take place that trigger the exposure of the N-terminal region (Ruffolo and Shore 2003). The exposure promotes the oligomerisation of Bax and Bak, leading to the formation of focal clusters on the MOM. Experiments using reconstituted systems have demonstrated that Bax and Bak can form supramolecular pores large enough to release cytochrome c (Saito et al. 2000; Kuwana et al. 2002; Esposti and Dive 2003).

Role of Bcl-2 in preventing apoptosis

The anti-apoptotic members of the Bcl-2 family play a prominent role in maintaining the integrity of the mitochondrial outer membrane in response to cytotoxic insult. Studies have demonstrated that Bcl-2 blocks the apoptotic pathway by preventing the release of apoptogenic factors such as cytochrome c from the mitochondria (Kluck et

al. 1997; Yang et al. 1997). In addition, Bcl-2 blocks upstream events prior to the MOMP including Bax/Bak activation, relocalisation, and oligomerisation (Mikhailov et al. 2001; Yi et al. 2003). Together, these findings support the hypothesis that Bcl-2 sequesters BH3-only proteins, preventing the allosteric activation of multidomain pro-apoptotic proteins that lead to MOMP (Cheng et al. 2001). Hence, the overexpression of Bcl-2 can render cells resistant to apoptosis by preventing the conduction of a death signal to the death effecter (Letai 2005b; Letai et al. 2005).

1.2.3 Antioxidant function of Bcl-2

Much of the research performed on Bcl-2 has focused on its role in heterodimerising pro-apoptotic members of the Bcl-2 family. However, the involvement of ROS in the regulation of apoptosis, led Hockenbery and colleagues to propose that Bcl-2 may function as an antioxidant (Hockenbery et al. 1993). As such, the anti-apoptotic activity of Bcl-2 may stem in part from its ability to enhance the antioxidant capacity of the cell. This theory was supported by the initial observation that Bcl-2 protected cells from apoptosis induced by the oxidants H_2O_2 and menadione (Hockenbery et al. 1993). In the same study, Bcl-2 overexpression suppressed lipid peroxidation and provided a similar level of protection from oxidant-induced cell death as the H_2O_2 scavenger NAC. Complementary findings later that year, provided further proof that Bcl-2 functioned by preventing the accumulation of ROS (Kane et al. 1993). Furthermore, the authors demonstrated that cells overexpressing Bcl-2 had higher levels of the antioxidant GSH; thus, providing a mechanism by which Bcl-2 could provide antioxidant defense.

The potential for Bcl-2 to function as an antioxidant was confirmed by the phenotypic assessment of Bcl-2 knockout mice (Nakayama et al. 1993; Veis et al. 1993). The Bcl-2 deficient mice developed pathologies consistent with defects in oxidative metabolism. For example, the mice exhibited conditions, such as hypopigmentation and polycystic kidney disease, which are associated with chronic oxidative stress (Lerchl 1994; Maser et al. 2002). Later studies with Bcl-2 deficient mice revealed higher levels of protein carbonyls in the brain, liver and kidneys, suggesting that such tissues are exposed to higher levels of ROS (Hochman et al. 1998; Hochman et al. 2000). In contrast, mice that overexpress Bcl-2 show a marked decrease in the

generation of ROS (Bogdanov et al. 1999), further implicating an antioxidant aspect to Bcl-2 function *in vivo*.

Since these early studies, numerous reports have demonstrated that Bcl-2 provides protection from oxidative stress (Kowaltowski and Fiskum 2005). In particular, the overexpression of Bcl-2 has been shown to decrease the production of ROS in response to apoptotic stimuli. For example, several groups have provided strong evidence that Bcl-2 overexpression prevents the accumulation of H_2O_2 in response to apoptotic stimuli (Kane et al. 1993; Esposti et al. 1999; Khar et al. 2003). Furthermore, Bcl-2 overexpression prevents the accumulation of reactive nitrogen species (Lee et al. 2001b), $O_2^{-\cdot}$ (Cai and Jones 1998) and $OH^{\cdot}$ (Amstad et al. 2001) in cells exposed to apoptotic triggers. Together, these reports suggest that Bcl-2 promotes survival by preventing ROS generation in response to death-inducing stimuli.

Consistent with these findings, several lines of evidence have shown that Bcl-2 overexpression protects cellular components such as lipids, protein and DNA from oxidative stress. For instance, Bcl-2 attenuates lipid peroxidation induced by a range of oxidants (Hockenbery et al. 1993; Fabisiak et al. 1997; Tyurina et al. 1997; Bruce-Keller et al. 1998; Lee et al. 2001a). In a similar manner, the overexpression of Bcl-2 reduces the amount of protein carbonyls seen both *in vitro* (Lee et al. 2001a; Lee et al. 2001b) and *in vivo* (Bogdanov et al. 1999). Bcl-2 overexpression can prevent DNA oxidation in some cell lines, but not others, suggesting that the antioxidant effect of Bcl-2 is cell-type specific (Deng et al. 1999; Lee et al. 2001b; Godley et al. 2002; Mandavilli et al. 2005).

Bcl-2 overexpression may provide protection from ROS by upregulating key antioxidant enzymes. Bcl-2 overexpression has been shown to increase the activity of Cu,Zn-SOD, catalase, and Gpx in various cell lines (Ellerby et al. 1996; Papadopoulos et al. 1998; Del Bufalo et al. 2001; Lee et al. 2001b); however, the phenomenon appears to be cell specific. For example, SOD and catalase activity were upregulated in Bcl-2 transfected PC12 cells but not GT1-7 cells (Ellerby et al. 1996). Likewise, Lee and colleagues reported that catalase activity was enhanced in Bcl-2 transfected SK-M-NC cells but not NT-2 cells (Lee et al. 2001b). The lack of any consistent

changes in antioxidant enzyme activities suggests that this may not be the primary mechanism by which Bcl-2 protects cells from ROS.

Several lines of evidence have shown that Bcl-2 overexpression enhances cellular redox by causing a concomitant increase in the level of GSH (Kane et al. 1993; Ellerby et al. 1996; Mirkovic et al. 1997; Meredith et al. 1998; Voehringer 1999; Lee et al. 2001b). The enhanced redox status in Bcl-2 overexpressing cells is associated with a resistance to apoptosis. Furthermore, Bcl-2 overexpressing cells can be re-sensitized to apoptosis by GSH depletion (Mirkovic et al. 1997; Rimpler et al. 1999; Armstrong and Jones 2002). These findings suggest that Bcl-2 may modulate apoptotic sensitivity through an antioxidant pathway that involves cellular thiols.

The Bcl-2-dependent change in redox status predictably increases the level of free protein thiols (Ellerby et al. 1996; Tyurin et al. 1998). An increase in reduced protein thiols is of great interest in light of recent data demonstrating that protein thiol oxidation can regulate the equilibrium between survival and apoptosis (Cross and Templeton 2004). In the generalised model, an increase in reduced thiols promotes survival, while specific oxidation of protein thiols leads to apoptosis. Consistent with this model, a DNA microarray analysis of Bcl-2 overexpressing cells revealed an increase in expression of enzymes involved in maintaining thiol homeostasis (Voehringer et al. 2000). Hence, the protective effect of GSH in Bcl-2 overexpressing cells may reflect a tendency for a specific protein thiol to remain in the reduced form, in spite of apoptotic stimuli that might promote its oxidation.

Recent studies have revealed that the overexpression of Bcl-2 leads not only to the elevation, but also the redistribution of GSH, from the cytosol to the nucleus (Voehringer et al. 1998). The importance of the nuclear GSH compartmentalisation is unknown; however, the authors propose that the redistribution of GSH may alter the activity of redox-sensitive transcription factors to promote cell survival.

Recent studies have greatly enhanced our understanding of how Bcl-2 may alter the antioxidant capacity when overexpressed in cultured cells (Kowaltowski and Fiskum 2005). The emerging view is that Bcl-2 overexpression may enhance the antioxidant status of cells via an adaptive response. This theory proposes that the overexpression

of Bcl-2 increases the volume and structural complexity of the mitochondria (Kowaltowski et al. 2002), which in turn affects the dynamics of the electron transport chain (Kowaltowski et al. 2000; Kowaltowski et al. 2004). These alterations augment the level of NADPH and increase the membrane potential (Esposti et al. 1999; Kowaltowski et al. 2000). The Bcl-2 dependent change in mitochondrial bioenergetics leads to an increase in the basal level of H_2O_2 production (Esposti et al. 1999; Armstrong and Jones 2002; Kowaltowski et al. 2004). Such findings are in agreement with the paradoxical reports that Bcl-2 can function as a pro-oxidant (Steinman 1995; Seyfried et al. 2003). The subtoxic increase in mitochondrial ROS may allow Bcl-2 overexpressing cells to adapt by upregulating antioxidant defenses.

Challenges to the antioxidant hypothesis

Various studies have challenged the hypothesis that Bcl-2 has an antioxidant function: instead, it is argued that Bcl-2 provides protection from apoptosis via a redox-independent mechanism. Several groups have demonstrated that Bcl-2 can protect cells from apoptosis in hypoxic conditions (Jacobson and Raff 1995; Shimizu et al. 1995). Such conditions would preclude the involvement of ROS and would, therefore, suggest that Bcl-2 blocks cell death by a ROS-independent mechanism. Related studies have shown that the protection Bcl-2 provides from apoptotic stimuli is independent of any alterations in cellular redox (Satoh et al. 1996; Gardner et al. 1997). Furthermore, the effect of Bcl-2 on both antioxidant enzymes and GSH metabolism is cell-line dependent (Ellerby et al. 1996; Schor et al. 2000; Lee et al. 2001b), arguing against a universal model in which Bcl-2 overexpression enhances the antioxidant capacity to promote cell survival.

1.3 Thesis Objectives

There are conflicting reports in the literature as to whether or not Bcl-2 acts as an antioxidant to augment the redox capacity of the cell, and whether this contributes to the anti-apoptotic function of Bcl-2. Many of the studies have been performed with only a single Bcl-2 transfected cell line. As such, they fail to provide a clear relationship between the level of Bcl-2 expression and the extent to which the redox

capacity of a cell is modified. This thesis aims to rectify this deficiency by employing a number of Bcl-2 transfectants with varying levels of Bcl-2 expression. The use of multiple transfectants for comparison ensures that any differences can be correlated to the level of Bcl-2 expression. Consequently, this study aims to determine the primary mechanism by which Bcl-2 protects Jurkat cells from H_2O_2.

The aims of this thesis are:

1) To characterize the ability of Bcl-2 to block cell death in Jurkat cells exposed to H_2O_2 (Chapter 3).

2) To determine the effect of Bcl-2 overexpression on the antioxidant status of Jurkat cells (Chapter 4).

3) To assess the extent of oxidative damage in Bcl-2 transfected Jurkat cells exposed to H_2O_2 (Chapter 5).

Chapter 2: Materials and Methods

2.1 Materials

Cell culture materials including RPMI 1640, fetal bovine serum (FBS), penicillin, streptomycin, and geneticin were from Gibco BRL, Invitrogen, Auckland, New Zealand.

The detergent compatible (DC) protein assay kit, 40% acrylamide/bis solution 37.5:1 and Bio-Spin® 6 chromatography columns were from Bio-Rad Laboratories (Hercules, CA, USA).

Rabbit polyclonal antibodies to peroxiredoxin 1-6, glutathione peroxidase 1-4, thioredoxin reductase 1 and 2, thioredoxin 1 and 2, glutaredoxin 1 and peroxiredoxin-SO_2H were all from Lab Frontier (Seoul, Korea).

Sheep anti-mouse IgG - horseradish peroxidase, Hybond-PVDF membrane and enhanced chemiluminescence (ECL™) Western blotting system were from Amersham Biosciences (Buckinghamshire, England).

C-11 BODIPY 581/591, 5-iodoacetamidofluorescein, BenchMark™ pre-stained protein ladder and biotinylated rabbit anti-dinitrophenyl-KLH polyclonal antibody were from Molecular Probes (Invitrogen, Auckland, New Zealand).

3[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) and Complete™ protease inhibitors were from Roche Diagnostics (Manheim, Germany).

Streptavidin-conjugated horseradish peroxidase and TMB+ chromagen were from Dako Corporation (Carpentria, CA, USA).

The artificial caspase substrate Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) was from the Peptide Institute Inc (Osaka, Japan).

Mouse anti-Bcl-2 was from Zymed Laboratories (San Francisco, CA, USA).

Human activating Fas antibody was from Upstate Biotechnology (Lake Placid, NY, USA)

The histological stain Diff-Quick^{TM} was from Baxter Healthcare Corporation (Miami, FL, USA).

The water-soluble tetrazolium dye (WST-1) was purchased from Dojindo Laboratories (Kumamoto, Japan).

All other chemicals and reagents were from Sigma Chemical Co (St Louis, MO, USA) and BDH Laboratory Supplies (Poole, England). All water was deionised and ultrafiltrated using a Milli-Q filtration system.

2.2 Cell Culture

Human Jurkat T lymphoma cell line derived from an acute lymphoblastic leukemia was acquired from the ATCC (Rockville, MD, USA) and grown in RPMI 1640 (Gibco BRL Life Technologies, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Jurkat transfectants overexpressing Bcl-2 and neo controls were generated in our laboratory by Dr Susan Thomson (section 3.2) and grown in RPMI 1640 supplemented with 10% FBS and 315 μg/ml geneticin. Cells were maintained in a humidified incubator at 37°C and 5% CO_2/air.

2.3 Treatment of cells

Cultured cells were collected at optimal growth (~0.7-1x10^6 cells/ml) and resuspended in fresh RPMI 1640 supplemented with 10% FBS at a concentration of 1x10^6 cells/ml (H_2O_2 treatment) or 5x10^6 cells/ml (Fas-treatment). Jurkat cells were transferred into either 6

well plates or 24 well plates, depending on the experimental requirements. Cells were left to equilibrate for one hour before being treated. H_2O_2 was made fresh daily by dilution of H_2O_2 stock solution in PBS. The concentration of H_2O_2 was determined by using the extinction coefficient ($\varepsilon_{240\ nm}$ = 43600 $M^{-1}.cm^{-1}$). Fas experiments were carried out using 250 ng/ml of agonistic Fas antibody.

2.4 Morphological assessment of treated cells

The morphology of treated cells was examined using an inverted phase contrast microscope (Olympus Optical Ltd, Tokyo, Japan) fitted with a 35 mm digital camera (Canon Powershot).

2.5 Cell Viability

Cell viability was measured with the DNA-intercalating vital dye propidium iodide (PI) (Hudson et al. 1969). PI is excluded from viable cells but is rapidly taken up by dead cells due to a loss of membrane integrity (Nicoletti et al. 1991). Treated cells ($5x10^5$) were harvested by centrifugation at 1000 *g* for 4 min and resuspended in a 5 ml plastic tube with 1 ml of PBS containing 5 μg PI. The cell suspension was incubated in the dark for 10 min and then 10,000 cells were analysed using a FACSCalibre flow cytometer (Becton Dickinson, Mountain View, CA) to determine the percentage of PI-positive cells.

2.6 Caspase assay

Caspase activity within treated cells was determined fluorometrically by following the cleavage of DEVD-AMC (Nicholson et al. 1995). Treated cells ($5x10^5$) were collected by centrifugation at 1000 *g* for 4 min and frozen down at –80°C until needed. Frozen pellets (containing approximately $5x10^5$ cells) were resuspended in 10 μl PBS and transferred to a 96 well plate. Ninety-five μl of caspase buffer (5 mM dithiothreitol, 100 mM HEPES, 10% sucrose, 0.1% NP-40 and 0.1% CHAPS at pH 7.25) containing 50 μM DEVD-AMC was added to the sample and the rate of AMC production (excitation 370 nm, emission 445 nm) was followed at 37°C with a POLARstar Galaxy fluorescent platereader (BMG Labtechnologies Pty. Ltd. Mt Eliza, Australia).

2.7 Preparation of cell lysates

Jurkat and Bcl-2 transfectant cell lysates were prepared by harvesting cells at optimal growth (cell concentration = 0.6-0.9x10^6 cells/ml) and subjecting them to centrifugation at 1000 *g* for 4 min. Cells were washed in PBS and resuspended in extract buffer (40 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% CHAPS, Complete™ protease inhibitors, and pH 7.4) at a concentration of 30 μl/10^6 cells. Lysates were incubated for 30 min at 4°C with sporadic vortexing. Thereafter, lysates were clarified by centrifugation at 12,000 *g* for 3 min. The resulting supernatant was stored at –80°C until required.

2.8 Protein assay

Protein concentrations were determined using the BioRad Detergent Compatible (DC) protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. A standard curve was obtained by transferring known concentrations (0-10 μg) of bovine serum albumin into a microplate along with 2 μl of sample lysates. 25 μl of reagent S was added to each well, followed by 200 μl of reagent B. The wells were thoroughly mixed and left for 15 min before the absorbances were read at 595 nm using a microplate spectrophotometer (MRX microplate reader, Dynatech Laboratories).

2.9 SDS-PAGE

Sample lysates were resolved for immunoblotting or fluorescence analysis by discontinuous SDS-PAGE (Laemmli 1970). Depending on the experimental procedure, 20-40 μg (mini-gel) or 50-100 μg (large-gel) of each sample lysate was combined with sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 5% β-mercaptoethanol) at a 2:1 ratio. Samples were loaded onto a 4% stacking gel with a molecular weight standard (BenchMark™ pre-stained protein ladder) and resolved with either a 12% or 15% gel in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) (Table 2.1). Mini SDS-PAGE was performed using the BioRad Mini Protean 3 system with a BioRad Powerpack 300 running for 90 min at 150 V. Large

format electrophoresis was run overnight on a Protean 2 Xi cell with an Ephortec 3000 power supply for 20 hrs at 20 mA.

Mini SDS-PAGE (2 gels)	**Separating Gel**			**Stacking Gel**
Gel %	**10%**	**12%**	**15%**	**4%**
Acrylamide (40% 37.5:1)	2.5 ml	3 ml	3.75 ml	1 ml
Water	5.4 ml	4.9 ml	4.15 ml	6.4 ml
2 M Tris HCl pH 8.8	2 ml	2 ml	2 ml	
0.5 M Tris HCl pH 6.8	-	-	-	2.5 ml
20% SDS	50 ul	50 ul	50 ul	50 ul
10% Ammonium persulfate (fresh)	50 ul	50 ul	50 ul	50 ul
TEMED	15 ul	15 ul	15 ul	15 ul
Large Format SDS-PAGE (2 gels)	**Separating Gel**			**Stacking Gel**
Gel %	**10%**	**12%**	**15%**	**4%**
Acrylamide (40% 37.5:1)	13.75 ml	15 ml	18.75 ml	2 ml
Water	29.7 ml	21.75 ml	20.75 ml	12.8 ml
2 M Tris HCl pH 8.8	11 ml	12.5 ml	10 ml	
0.5 M Tris HCl pH 6.8	-	-	-	5 ml
20% SDS	225 ul	250 ul	250 ul	100 ul
10% Ammonium persulfate (fresh)	225 ul	250 ul	250 ul	100 ul
TEMED	25 ul	25 ul	25 ul	20 ul

Table 2.1: SDS-PAGE gels were made using the standard recipe summarised above.

2.10 Immunoblot detection of proteins

Immunoblotting was performed as previously described (Burnette 1981). The protein concentration of cell lysates was determined by the DC protein assay. After addition of 20 μl sample loading buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 5% β-mercaptoethanol), protein extracts (20-40 μg) were analysed by SDS-PAGE. Proteins were transferred from SDS-PAGE gels to Hybond PVDF membranes by Western blotting in blot buffer (25 mM Tris, 192 mM glycine, 10 % methanol) for 50 min at 100 Volts. The PVDF membranes were blocked in 5% skim milk $TBST_{20}$ (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.05% Tween 20) for an hour before being washed and probed with the appropriate primary antibody in 2% skim milk $TBST_{20}$ overnight at 4°C (Table 2.2). The next day, the blots were washed thoroughly in $TBST_{20}$ and probed with either HRP-conjugated sheep anti-mouse (1:20,000 dilution) or HRP-conjugated goat anti-rabbit (1:20,000 dilution) antibodies in 2% skim milk $TBST_{20}$ for an hour. After incubating with the secondary antibody, the membranes were washed for the final time in $TBST_{20}$. Antibodies bound to the PVDF

membrane were detected using an enhanced chemiluminescence system (ECL) (Amersham Biosciences) and visualized using Kodak film or the Chemidoc FX (BioRad laboratories, Hercules, CA, USA).

Antibody	Dilution	Secondary detection
anti-Bcl-2	1:20,000	HRP-conjugated sheep anti-mouse
anti-GAPDH	1:20,000	HRP-conjugated sheep anti-mouse
anti-actin	1:5,000	HRP-conjugated sheep anti-mouse
anti-glutathione peroxidase 1	1:2,000	HRP-conjugated goat anti-rabbit
anti-glutathione peroxidase 3	1:2,000	HRP-conjugated goat anti-rabbit
anti-glutathione peroxidase 4	1:2,000	HRP-conjugated goat anti-rabbit
anti-peroxiredoxin 1	1:5,000	HRP-conjugated goat anti-rabbit
anti-peroxiredoxin 2	1:20,000	HRP-conjugated goat anti-rabbit
anti-peroxiredoxin 3	1:5,000	HRP-conjugated goat anti-rabbit
anti-peroxiredoxin 4	1:2,000	HRP-conjugated goat anti-rabbit
anti-peroxiredoxin 5	1:2,000	HRP-conjugated goat anti-rabbit
anti-peroxiredoxin 6	1:2,000	HRP-conjugated goat anti-rabbit
anti-thioredoxin 1	1:1,000	HRP-conjugated goat anti-rabbit
anti-thioredoxin reductase 1	1:2,000	HRP-conjugated goat anti-rabbit
anti-thioredoxin reductase 2	1:2,000	HRP-conjugated goat anti-rabbit
anti-peroxiredoxin-SO2H	1:2,000	HRP-conjugated goat anti-rabbit

Table 2.2: Primary antibody dilutions and requirements for secondary detection.

2.11 Superoxide dismutase assay

Superoxide dismutase (SOD) activity was measured according to the method of Peskin et al. (Peskin and Winterborn 2000). The assay uses a superoxide generating system (xanthine oxidase and hypoxanthine) to reduce a water-soluble tetrazolium dye (WST-1). The reduction of WST-1 was followed kinetically at 450 nm. The presence of SOD in the system prevents WST-1 reduction. A dilution series of purified SOD (0-12 ng/ml) and sample lysates (0-8 μg) were transferred to a 96-well microplate. Undiluted sample lysate aliquots (50 μg) were also transferred to the microplate in order to reveal the maximal inhibition of WST-1 reduction. The microplate samples were mixed with 200 μl of a freshly prepared reaction cocktail containing 2.5 μl of 1.38 mg/ml xanthine oxidase, 100 μl of 10 mM WST-1, and 100 μl of 2 mg/ml catalase diluted in 19.3 mls of SOD buffer (50 mM sodium phosphate buffer, 0.1 mM DTPA and 0.1 mM hypoxanthine at pH 8). The reaction was followed over 5 min by monitoring the change in absorbance at 450nm (MRX microplate reader, Dynatech Laboratories). Changes in $A_{450\ nm}$ were plotted against the amount of sample, to produce a curve describing the inhibition of WST-1

reduction in the presence of SOD. The SOD activity of each lysate was determined by finding the amount of sample required to inhibit WST-1 reduction by 50% (=1 Unit SOD activity). “Cyanide-insensitive” SOD activity was measured in the presence of 5 mM sodium cyanide, which was added to the reaction mix just prior to the assay.

2.12 Catalase assay

The catalase activity in sample lysates was determined as previously described (Aebi 1984), by following the decomposition of H_2O_2 at 240nm. Ten μg of sample lysate was added to 1 ml of 12 mM H_2O_2 (Initial $A_{240\ nm}$ ~ 0.45 au) in a quartz cuvette and the reaction was followed on an Agilent UV-Vis spectrophotometer for 2 min to give a linear rate of decomposition (au/min). The reaction was stopped with sodium azide (1 mM) to confirm that the H_2O_2 decomposition was catalase-dependent. Decomposition rates were calculated from a standard curve, formed by using purified catalase, to determine the relative catalase activity (U/mg) of each sample lysate.

2.13 Glutathione peroxidase assay

Glutathione peroxidase activity was measured according to the protocol of Flohe et al. (Flohe and Gunzler 1984). The method involves coupling the H_2O_2-decomposing activity of glutathione peroxidase to generate oxidised glutathione, with glutathione reductase, which consumes NADPH. The decomposition of NADPH was followed over time on a spectrophotometer at 340 nm. Five μg of sample lysate was added to 1 ml of a freshly prepared reaction cocktail (48 mM sodium phosphate, 0.38 mM EDTA, 0.95 mM sodium azide, 1 mM glutathione, 0.02 mM DTT, and pH 7) in a quartz cuvette. Glutathione reductase (3.2 units) and β-NADPH (0.12 mM) were then added to the cocktail and mixed by inversion. Finally, H_2O_2 (250 μM) was combined with the reaction mixture and the decrease in absorbance at 340 nm was monitored on an Agilent UV-Vis spectrophotometer for 5 min. The $\Delta A_{340\ nm}$ was used to ascertain the glutathione peroxidase activity (U/mg) of each sample.

2.14 Thioredoxin reductase assay

The activity of thioredoxin reductase was measured using minor modifications to the method of Arner et al. (Arner et al. 1999). The assay is based on the principle that thioredoxin reductase reduces 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) in an NADPH-dependent manner. Sample lysates (50 μg) were transferred to a microplate and diluted with extract buffer (40 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% CHAPS, Complete™ protease inhibitors, and pH 7.4) to a concentration of 1 mg/ml. The microplate samples were mixed with 50 μl of 10 mM DTNB and the change in absorbance at 412 nm was monitored for 2 min to give a baseline DTNB reduction. After this, 10 μl of 2 mM NADPH was added to the reaction mix in order to determine the NADPH-dependent DTNB reduction. The relative activity of thioredoxin reductase was equivalent to the difference between $\Delta A_{412\ nm}$ before and after the addition of NADPH.

2.15 Determination of thiols

Free thiol groups in cell lysates were quantified using Ellman's reagent (DTNB) as previously described (Ellman 1958). Fifty μl aliquots of cell lysates (1 mg/ml) and 1-10 μl aliquots of 1 mM glutathione (1-10 μM) were transferred into a 96-well microplate. The samples were mixed with 100 μl of DTNB (200 μM final concentration) in PBS and incubated for 15 min in the dark. After this period of time, the absorbances were read at 412 nm using a MRX microplate reader (Dynatech Laboratories). The concentration of thiols was determined by using the extinction coefficient for DTNB ($\varepsilon_{412\ nm}$ = 13600 $M^{-1}cm^{-1}$.).

2.16 Ferrous oxidation of xylenol orange (FOX) assay

The level of H_2O_2 present in cell culture medium was determined by the FOX assay as described by Wolff et al. (Wolff 1994). The FOX assay is based on the principle that H_2O_2 in solution oxidises Fe^{2+} to Fe^{3+}, which can then complex with xylenol orange to form a purple product that can be quantified on a spectrophotometer. Cell culture aliquots (10-50 μl) were transferred into 1 ml of FOX reagent (100 μM xylenol orange, 250 μM $Fe(NH_4)SO_4$, 100 mM sorbitol, 25 mM H_2SO_4), vortexed and left for 40 min. A standard

curve was produced in parallel by mixing 1-5 μl aliquots of 1 mM H_2O_2 into 1 ml of FOX reagent. After incubation, samples were transferred into spectrophotometer cells and absorbances were measured at 560 nm on an Agilent UV-Vis spectrophotometer. Unknown H_2O_2 concentrations were determined by comparison to the standard curve.

2.17 IAF-labeling of reduced thiols

Protein-thiols in cell lysates were fluorescently labeled with 5-iodoacetamidofluorescein (5-IAF) (Molecular Probes Inc, Eugene, OR). 5-IAF is a fluorescent probe that specifically reacts with low pKa thiols to give a fluorescent adduct. Fifty μg of cell lysate was incubated with 200 μM 5-IAF for 15 min in the dark. Excess 5-IAF was removed from the samples by passing the extracts through Bio-Spin® 6 chromatography columns (Bio-Rad Laboratories, Hercules, CA) pre-equilibrated with extract.

2.18 IAF-labeling of oxidised thiols

Reversibly oxidised thiol-containing proteins were fluorescently labeled using a technique developed by Baty et al. (Baty et al. 2002; Baty 2004) (Figure 2.1). The technique involved blocking reduced protein thiols by alkylation with N-ethylmaleimide (NEM). Oxidised thiols were then reduced with dithiothreitol (DTT) and labeled with 5-IAF. Cell samples ($5x10^6$ cells) were harvested by centrifugation at 1000 *g* for 4 min, washed in PBS, then resuspended in 75 μl NEM buffer and incubated on ice for 15 min. The cells were solubilised with 1% CHAPS for 15 min and centrifuged at 16,000 g for 4 min in order to pellet cell debris. The clarified extracts were desalted using Bio-Spin® 6 chromatography columns equilibrated with extract buffer. The resulting lysates were treated with 1 μl of 1 M DTT for 10 min, followed by 2 μl of 10 mM 5-IAF for a further 15 min in the dark. After incubation, excess 5-IAF was removed from the samples by passing extracts through a Bio-Spin® 6 column equilibrated with extract buffer.

2.19 Detection of 5-IAF-derivatised protein thiols

The protein concentration of each 5-IAF labeled lysate was determined before SDS-PAGE to ensure equal protein loading. 5-IAF labeled samples were resolved by SDS-

PAGE and then visualized using a BioRad FX fluorescence scanner (excitation λ=488 nm and emission λ=530 nm, Bio-Rad Laboratories, Hercules, CA, USA).

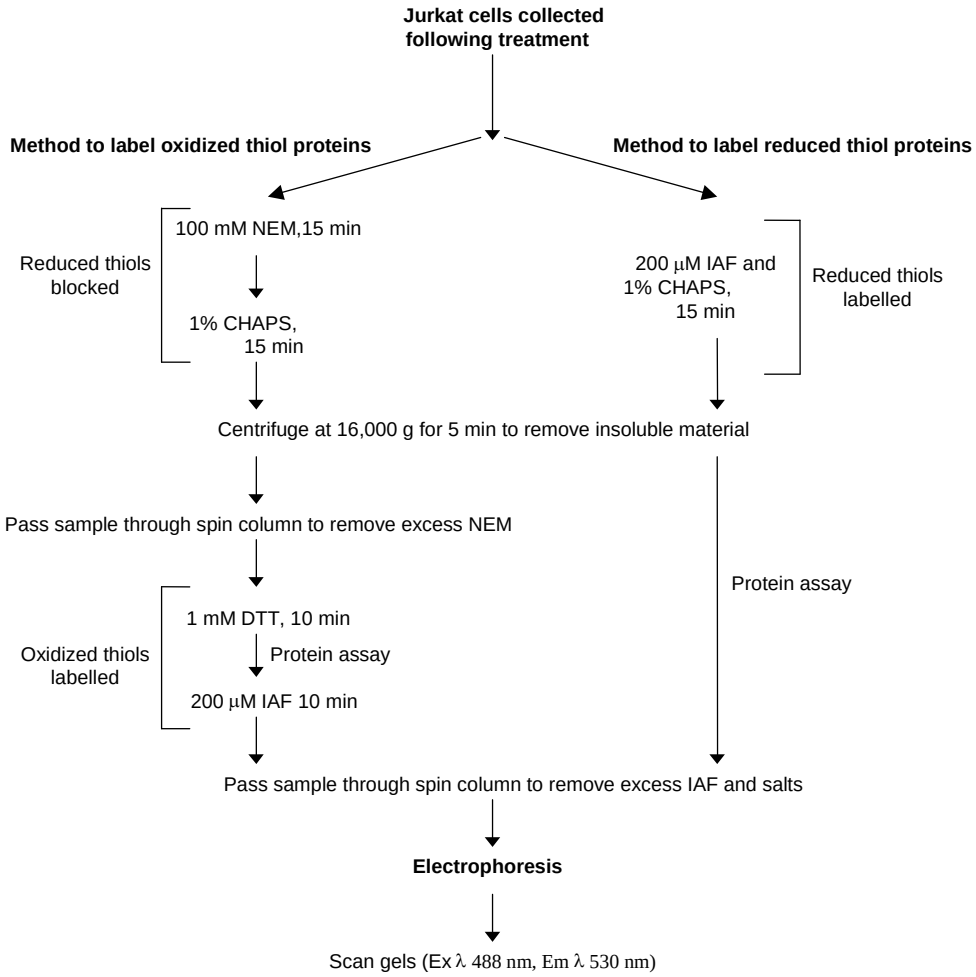


Figure 2.1: Scheme for the fluorescent labelling of oxidised and reduced thiol proteins in treated Jurkat cells *(adapted from Baty 2004).*

2.20 Coomassie Staining

To assess the amount of protein loaded into each lane, gels were stained with Coomassie blue protein stain. Gels were stained overnight in 0.1% Coomassie Blue G250 containing 20% methanol. The following day, gels were de-stained with multiple changes of water and imaged using a BioRad Fluor-S™ MultiImager (Bio-Rad Laboratories, Hercules, CA, USA).

2.21 Silver Staining

SDS-PAGE gels were fixed overnight in 50% ethanol and 10% acetic acid. The next day the gels were immersed in oxidising solution (0.2 g/l anhydrous sodium thiosulfate) for 10 min followed by silver stain (2 g/l $AgNO_3$ + 750 μl/l formaldehyde) for 30 min. Thereafter, gels were developed for up to 10 min in developing solution until appropriate colour development was reached (60 g/l sodium carbonate, 500 μl/l formaldehyde and 20 mls oxidising solution) before the reaction was stopped with stop solution (20 g/l sodium EDTA). Gels were washed thoroughly between each step with ddH_2O and imaged using a BioRad Fluor-S™ MultiImager (Bio-Rad Laboratories, Hercules, CA, USA).

2.22 Lipid Peroxidation assay

Lipid peroxidation was detected by utilising the oxidation-sensitive lipophilic probe BODIPY 581/591 C-11, as previously described (Drummen et al. 2002). Upon oxidation the BODIPY C-11 fluorescence emission peak shifts from ~590 nm (red) to ~510 nm (green). Cells were incubated for 30 min in media containing 5 μM BODIPY C-11. After incubation, cells were washed in PBS and resuspended in fresh RPMI 1640 supplemented with 10% FBS. Resuspended cells were then treated with H_2O_2 for an hour before being analysed by flow cytometry (FACSCalibre flow cytometer, Becton Dickinson, Mountain View, CA). The change in intensity of the BODIPY-Green fluorescent signal was used to determine the extent of lipid peroxidation.

2.23 Protein Carbonyl ELISA

Protein carbonyls were detected using a microplate ELISA kit developed in our laboratory (Buss et al. 1997). The method involved derivatising protein carbonyls with dinitrophenylhydrazine (DNP) and adsorbing them to a hi-bond microplate. The DNP-derivitised protein bound to the microplate was detected using an ELISA based assay. Treated cells ($1x10^6$) were collected by centrifugation at 1000 *g* for 4 min and lysed in 40 μl of PBS by freeze-thaw cycling (Note: detergents and Complete™ protease inhibitors interfere with the assay). The protein concentration of sample lysates was determined and then 20 μg of protein was transferred to an Eppendorf and dried using a speed-vac centrifuge equipped with a refrigerated vapor trap (Savant, Global Medical

Instrumentation, Minnesota, USA). The sample pellets were resuspended in 5 μl of PBS and derivatised with 15 μl of 0.1 mM DNP for 45 min at room temperature. Carbonyl kit standards and quality controls were diluted 10-fold and derivatised in parallel with the samples. After incubation, 5 μl of each derivatised sample or standard was mixed with 1 ml of PBS and immediately transferred (200 μl/well) in triplicate to a hi-bond ELISA plate (Corning Costar). The microplate samples were left overnight at 4°C to allow time for the protein to adsorb to the well plate. The following day, plates were washed in PBS-$Tween_{20}$ (0.05%) and probed with biotinylated anti-DNP (1:1000) in PBS-$Tween_{20}$ (0.05%) for 60 min. After washing again with PBS-$Tween_{20}$ (0.05%), the microplate was incubated with streptavidin-conjugated horseradish peroxidase (1:5000) in PBS-$Tween_{20}$ (0.05%) for 60 min. After a final wash with PBS-$Tween_{20}$ (0.05%), 200 μl of chromagen (TMB+) was added to each well and incubated until colour developed, at which point, 80 μl of stop solution (2.5 M H_2SO_4) was added and the absorbances read at 450 nm using a spectrophotometer plate reader (MRX microplate reader, Dynatech Laboratories). The carbonyl content of each sample was determined by comparing to the standard curve.

2.24 Fluorescence Microscopy

The lipid peroxidation assay was adapted so that cells could be assessed microscopically. The staining methodology was similar; however, stained cells were resuspended at a concentration of $5x10^5$ cells/ml in PBS and transferred to a glass slide by cytocentrifugation at 300 *g* for 5 min. The slides were air-dried, fixed in 4% paraformaldehyde for 10 min, and coated with Vectorshield mounting medium for fluorescence microscopy (Vector laboratories Inc, Burlingame, CA, USA) before being imaged with a fluorescent microscope.

2.25 Cell Growth

Cell growth was monitored by counting cells on a hemocytometer using an inverted phase contrast microscope (Olympus Optical Ltd, Tokyo, Japan). Jurkat cells were grown for a week and the number of viable cells was determined on a daily basis by using the vital dye trypan blue. Each day, 50 μl of cell culture was mixed with 50 μl of 0.5% trypan blue and then 10 μl of the mixture was transferred to a hemocytometer for

examination. The trypan blue dye specifically stains dead cells but is actively excluded from live cells. Cell culture medium was replaced on a daily basis, by pelleting cells and resuspending in fresh RPMI 1640 supplemented with 10% FBS, to ensure optimal growth conditions.

2.26 Cytokinesis Block Micronuclei (CBMN) assay

The CBMN assay was performed as described by Fenech et al. (Fenech 2000; Fenech et al. 2003). The principle behind the assay is to block cells in telophase and scan the resulting binucleate (BN) cells for nuclear blebs known as micronuclei (MN). The presence of micronuclei in treated cells is a marker of genomic instability. Cells were challenged with different doses of H_2O_2 for 4 hr before being cultured for a further 24 hr in media containing 4.5 μg/ml cytochalasin B. The next day, cells were harvested by centrifugation at 1000 *g* for 4 min and resuspended in PBS to a concentration of $5x10^5$ cells/ml. A 120 μl aliquot of each sample was transferred to a glass slide by cytocentrifugation at 500 rpm for 5 min. The slides were air-dried, fixed in absolute methanol for 10 min and stained using Diff-Quick. At least 1000 BN cells were counted on each slide. Micronuclei were scored (number of micronuclei / 1000 binucleate cells) using the criteria previously described under an oil immersion lens (Fenech et al. 2003).

2.27 Statistics

Values are shown as the mean $\pm$ the standard error of at least 3 individual experiments. Correlations are tested for significance using Pearson's paired comparison of variables. All statistical analyses were performed with the software package Sigmastat (Jandel Scientific, San Rafael, USA)

Chapter 3: Characterising the response of Bcl-2 transfected Jurkat T cells to H_2O_2

3.1 Introduction

H_2O_2 can elicit a variety of physiological responses including proliferation, temporary growth arrest, cellular senescence, apoptosis and necrosis, depending on the dose (Davies 1999). Sub-lethal doses of H_2O_2 have a mitogenic effect, activating gene expression to induce proliferation (Burdon 1995). At such doses, H_2O_2 has been proposed to act as a signalling molecule, intentionally generated by NADPH oxidases in response to receptor activation (Finkel 2000). Greater concentrations of H_2O_2 stimulate an adaptive response, in which cells become transiently or permanently growth arrested (Ben-Porath and Weinberg 2005). The cytotoxic activity of H_2O_2 has been well documented in a variety of mammalian cell types, including the Jurkat T lymphoma cell line (Khanna et al. 1998; Chiaramonte et al. 2001; Barbouti et al. 2002). H_2O_2 can trigger two distinct forms of cell death, depending on the level of oxidant exposure (Hampton and Orrenius 1997, 1998; Chandra et al. 2000; Troyano et al. 2003). Intermediate levels of H_2O_2 exposure are associated with apoptotic cell death, while higher doses of H_2O_2 promote necrosis. The switch from apoptosis to necrosis is mediated by both the oxidative inactivation of caspases (Hampton and Orrenius 1997; Hampton et al. 2002) and the depletion of ATP (Eguchi et al. 1997). ROS, such as H_2O_2, are thought to promote apoptosis primarily by oxidising cellular thiols (Cross and Templeton 2004). In support of this model, antioxidants that maintain thiols in their reduced state, including GSH and N-acetyl cysteine, prevent apoptosis (Um et al. 1996; Devadas et al. 2003; Cross and Templeton 2004).

Previous studies have shown that Bcl-2 overexpression renders cells resistant to H_2O_2 (Hockenbery et al. 1993; Zhong et al. 1993; Ichimiya et al. 1998; Rimpler et al. 1999; Ogidigben et al. 2000; Chiaramonte et al. 2001; Lee et al. 2001; Godley et al. 2002; Chen et al. 2003). It is not clear whether Bcl-2 protects the cells from H_2O_2 by enhancing the decomposition of H_2O_2 and the repair/removal of oxidised cellular

constituents, or if Bcl-2 prevents cells from undergoing apoptosis induced by oxidative stress. Many studies have revealed that Bcl-2 promotes a thiol rich intracellular environment resistant to the cytotoxic actions of ROS such as H_2O_2 (Rimpler et al. 1999; Lee et al. 2001; Jang and Surh 2004; Kowaltowski and Fiskum 2005). In contrast, other investigators have found that Bcl-2 provides no protection from H_2O_2-induced cell death (Miyashita and Reed 1992; Lee and Shacter 1997). The aim of this series of experiments was to further characterise the protection Bcl-2 provides against the oxidant H_2O_2 using transfected Jurkat T lymphocytes.

The major objectives of this chapter were:

1) To determine the concentrations of H_2O_2 that trigger apoptosis and necrosis in Jurkat cells.

2) To determine the effect of Bcl-2 overexpression on H_2O_2-induced apoptosis and necrosis in Jurkat cells.

3.2 Experimental approach

The following experiments were performed with a Jurkat T lymphoma cell line derived from an acute lymphoblastic leukemia (Gillis and Watson 1980). In order to study the effect of Bcl-2 overexpression in cultured cells, a number of Bcl-2 transfected Jurkat T lymphocytes were generated by Dr Susan Thomson. The cells were transfected via lipofection with a pCI-neo vector (Invitrogen, Auckland, New Zealand) (Brondyk 1994) containing the BCL-2 cDNA insert (kindly donated by Dr Suzanne Cory, Melbourne, Australia) (Figure 3.1). Stable Bcl-2 transfectants were selected in geneticin for 4 weeks and cryogenically preserved until required. Neo transfectants, carrying the vector without the insert, were generated in parallel to provide a set of control transfectants that had undergone the same selection procedure. Seven transfectants and the parental Jurkat cell line were chosen for further study. Two empty vector Neo transfectants (N1 and N2) and five Bcl-2 transfectants (B1, B2, B9, B36 and B38) were utilised. The relative level of Bcl-2 in the transfected cell lines was determined by immunoblotting for Bcl-2.

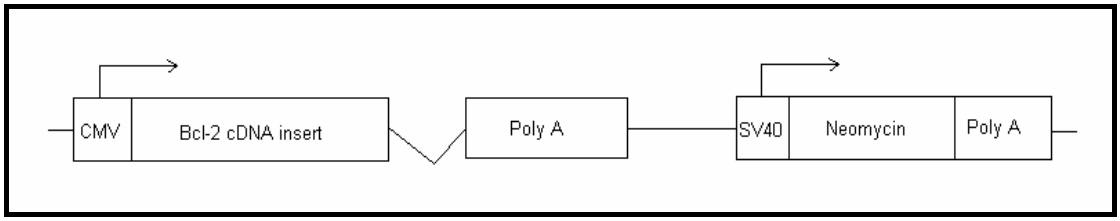


Figure 3.1: Diagram of the pCI-neo construct containing the Bcl-2 cDNA insert.

Jurkat cells and Bcl-2 transfectants were treated with Fas or H_2O_2 and a number of cell parameters were measured. Morphological alterations were detected by examination of cells under a light microscope. Apoptotic cells activate caspase-3-like effector caspases, which cleave various cellular proteins at conserved Asp-Glu-Val-Asp (DEVD) motifs (Thornberry et al. 1997). Caspase-3 activity was measured by monitoring the cleavage of the fluorogenic substrate DEVD-AMC over time (Nicholson et al. 1995). Caspases were inhibited by using the pan-caspase inhibitor z-VAD.fmk (1mM), which directly inhibits the proteolytic activity of the caspases (Slee et al. 1996). Cell viability was measured by using the vital dye propidium iodide (PI) and fluorescence-activated cell sorting (FACS) flow cytometry (Nicoletti et al. 1991). The membrane integrity of dead cells is compromised, allowing PI to enter and intercalate with the major groove of DNA to form a red fluorescent adduct (Hudson et al. 1969). PI-stained cells were subjected to FACS analysis to determine the proportion of dead cells (PI-positive) in a treated population.

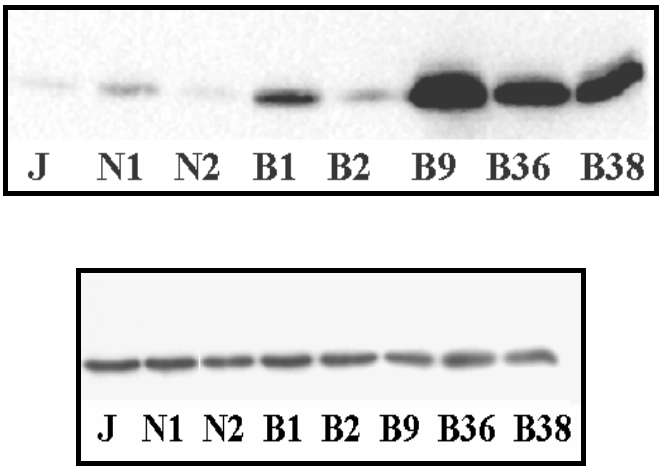
3.3 Results

3.3.1 Determining the relative level of Bcl-2 present in transfected cell lines

The transfectants utilised in this study showed a wide range of Bcl-2 expression (Figure 3.2A and B). The B1 and B2 clones had a low level of Bcl-2 expression, similar to controls (Jurkat, N1 and N2). Surprisingly, the N1 clones displayed a 9-fold increase in Bcl-2 expression, despite being transfected with an empty pCI-neo vector. It would appear that the increase in endogenous Bcl-2 expression seen in the N1 clone is an artifact of the transfection procedure. This clone was included in subsequent analyses to enable comparison between endogenous and vector Bcl-2 overexpression. The B36 and B38 clones displayed a moderately elevated level of Bcl-2 expression:

about 30-fold greater than controls. The B9 clone showed the greatest increase in Bcl-2 expression with approximately 60 fold greater expression of Bcl-2 than control cells.

A)



B)

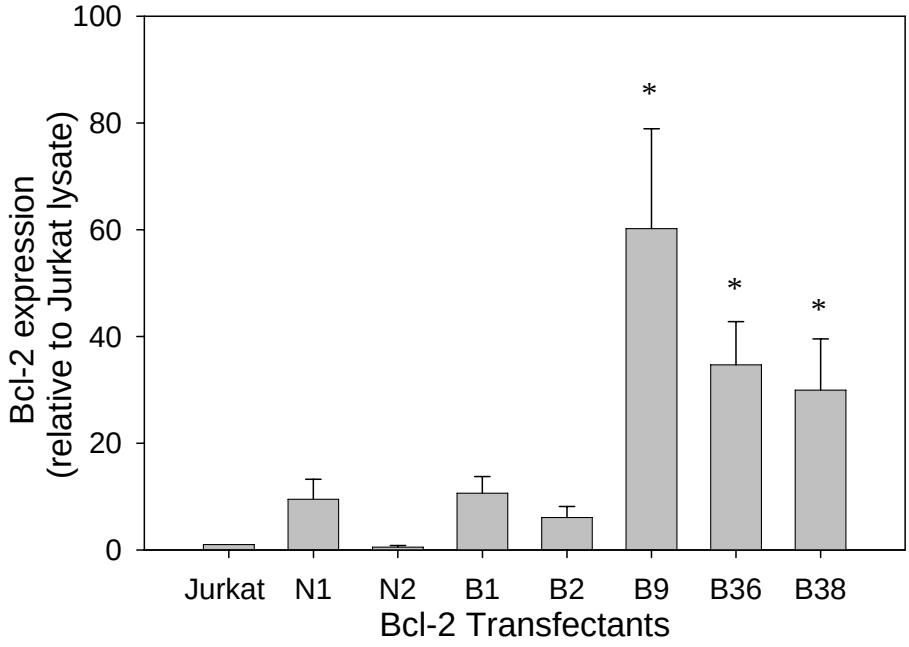
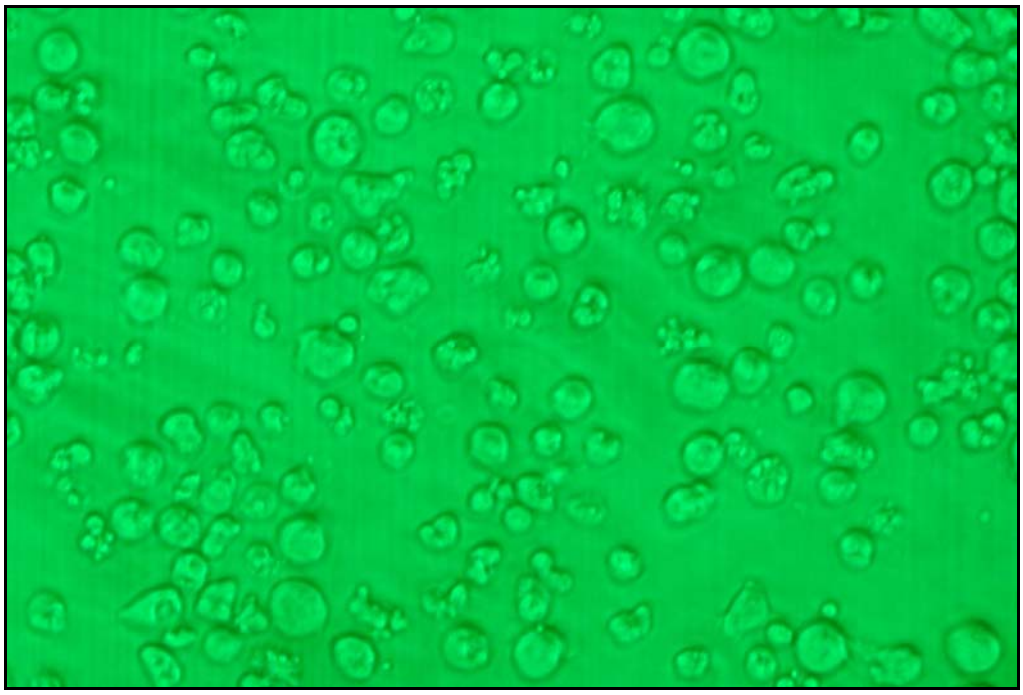


Figure 3.2: Bcl-2 expression levels in Bcl-2 transfected cells*. (A) Illustrative Western blot of 3 independent experiments showing Bcl-2 expression in transfectants. Transfected cell lysates were prepared from cultures in the exponential phase of growth. Samples were then resolved by SDS-PAGE and immunoblotted for Bcl-2. As a loading control, all samples were further blotted for GAPDH, a high abundance housekeeping protein. (B) Densitometry histogram of Bcl-2 blots. The bands were quantified using Quantity-One (BioRad). Values represent the mean ± SE of 3 independent experiments. * Indicates a significant difference ($p<0.05$) from Jurkat cells as determined by a One Way Repeated Measures ANOVA with Bonferroni multiple comparison (SigmaStat).*

3.3.2 Testing Jurkat and B9 cells against a classical apoptotic stimulus: Fas-mediated apoptosis

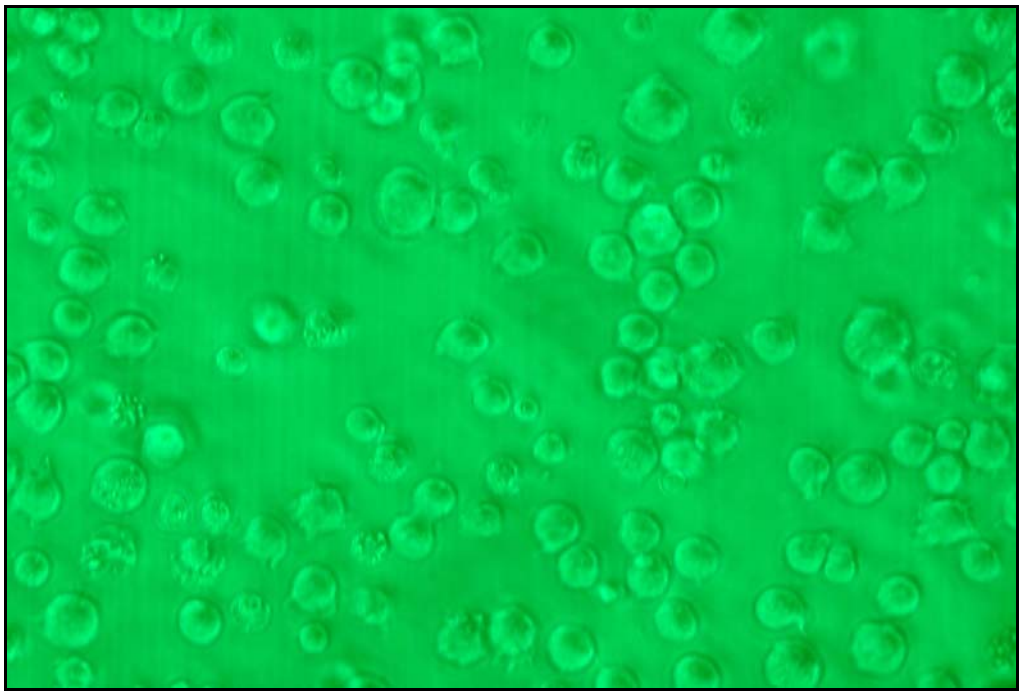
Prior to characterising the effect of H_2O_2 on the Bcl-2 transfectants, Jurkat and B9 clones were challenged with the agonistic Fas antibody: a classical stimulator of Fas-mediated apoptosis. Fas-stimulated Jurkat cells displayed typical apoptotic morphology, including cell shrinkage and membrane blebbing (Figure 3.3A). Furthermore, the apoptotic Jurkat cells experienced a time-dependent increase in the activity of the cell death protease caspase-3 (Figure 3.4A). The caspase activity peaked with a 25-fold increase three hr after Fas-stimulation. In contrast, the Fas-stimulated B9 clones exhibited very little apoptotic morphology (Figure 3.3B). Consistent with these observations, the B9 clones had a low level of caspase-3 activity (Figure 3.4B).

A)



Jurkat + Fas

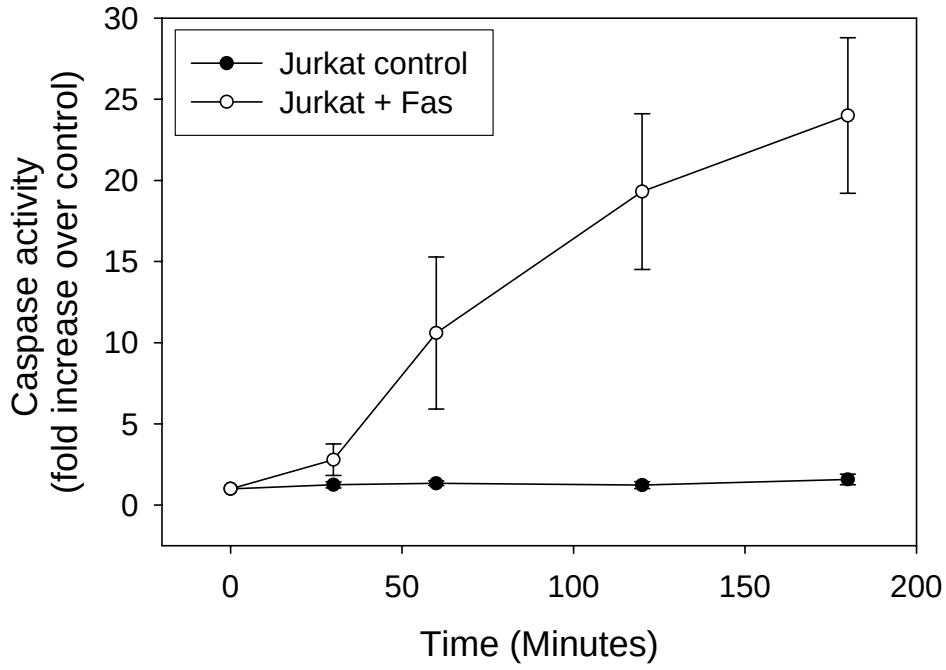
B)



B9 + Fas

Figure 3.3: Morphology of Jurkat (A) and B9 (B) cells treated with Fas antibody. *Cells were treated with 250 ng/ml activating human anti-Fas antibody for 2 hr. Images are representative of three independent experiments.*

A)



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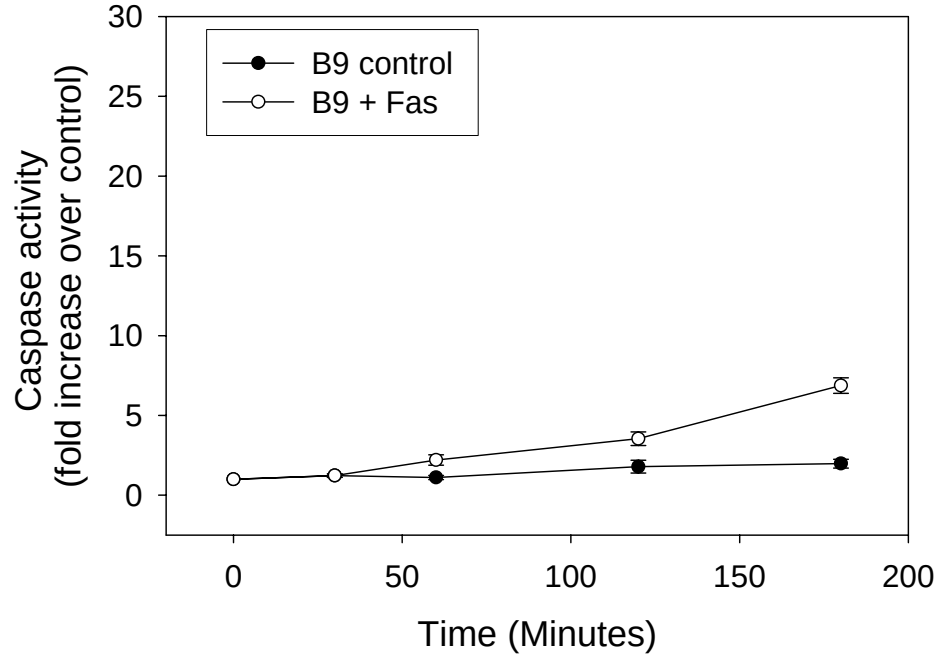


Figure 3.4: Caspase-3 activity in Fas treated Jurkat and B9 cells. *Jurkat (A) and B9 (B) cells were treated with and without 250 ng/ml of activating human anti-Fas antibody. Cell samples were removed periodically and assayed for caspase-3 activation by monitoring cleavage of DEVD-AMC. Values represent the mean ± SE of 3 independent experiments.*

3.3.3 Effect of H_2O_2 on Jurkat cell viability

The viability of H_2O_2-treated Jurkat cells was determined for a range of H_2O_2 concentrations (Figure 3.5). The Jurkat cells were vulnerable to H_2O_2, with over 50% loss of cell viability (LD_{50}) when challenged with 50 μM H_2O_2. The entire Jurkat cell population was killed by exposure to 200 μM H_2O_2. The mode of cell death (apoptosis or necrosis) could not be determined by this assay, hence it was important to examine the cellular morphology and caspase activity when exposed to different doses of H_2O_2. Jurkat cells treated with low concentrations of H_2O_2 (50 μM H_2O_2) displayed membrane blebbing characteristic of apoptosis (Figure 3.6). However, at higher concentrations of H_2O_2 (200 μM H_2O_2), the Jurkat cells became swollen and necrotic before shrinking at later time points (Figure 3.6).

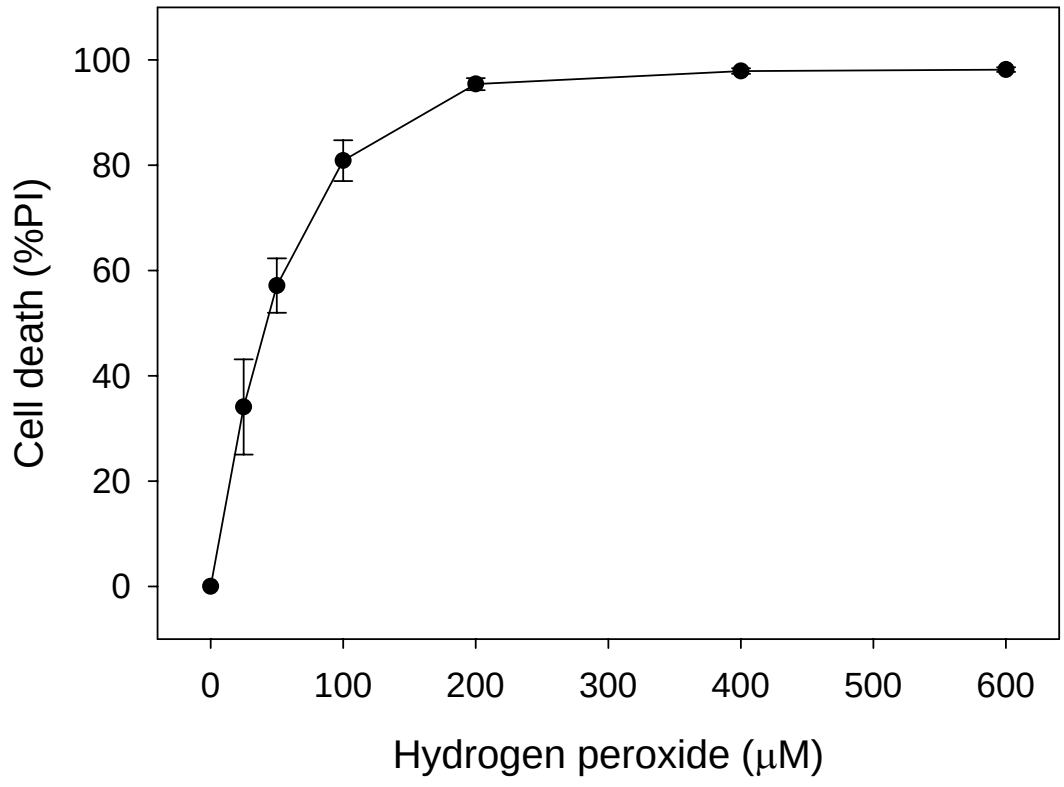


Figure 3.5: Cell viability of Jurkat cells exposed to H_2O_2. *The Jurkat cells were challenged with a range of H_2O_2 doses and incubated for 24 hr. Cells were subsequently stained with PI and subjected to FACS analysis to differentiate the proportion of PI positive cells in the population. Values represent the mean ± SE of 4 independent experiments.*

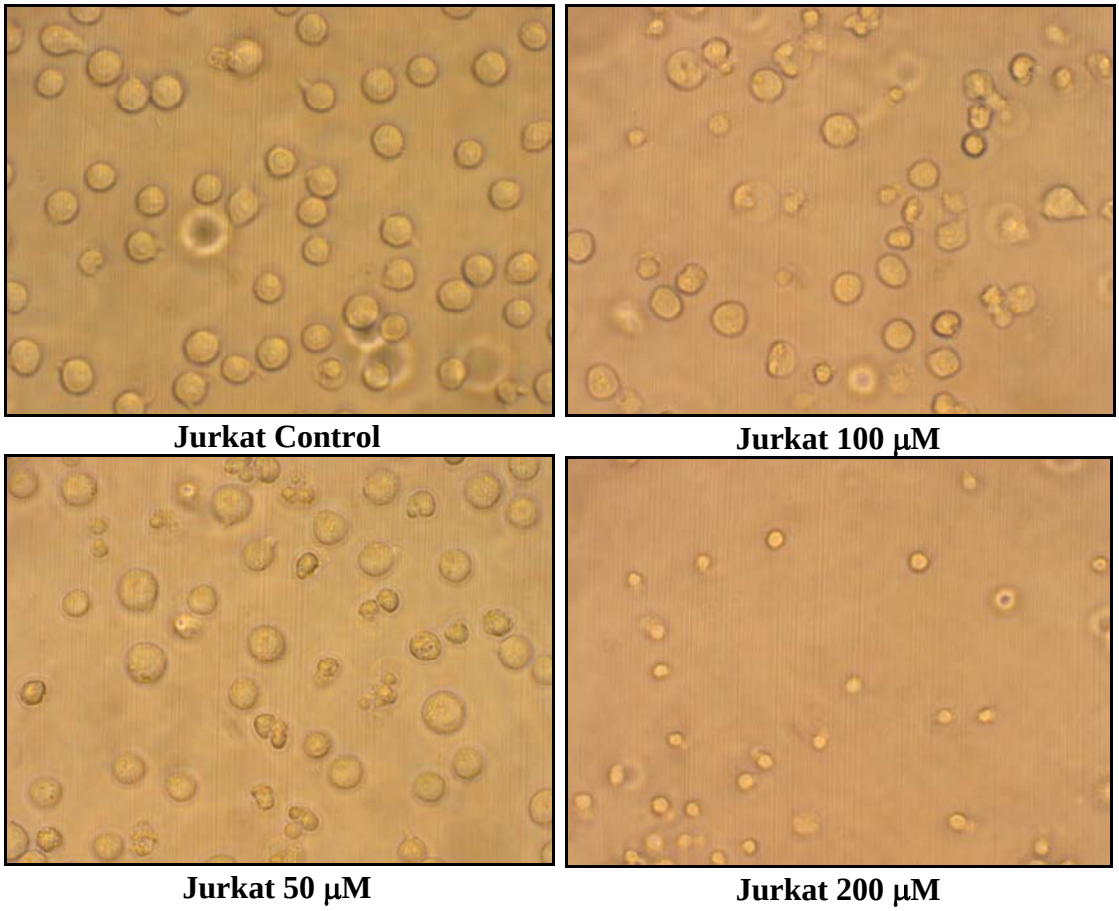


Figure 3.6: Assessing the morphology of Jurkat cells treated with H_2O_2. *Cells were treated with H_2O_2 for 6 hr. Photographs are representative of 3 independent experiments.*

3.3.4 Effect of H_2O_2 on Jurkat caspase activity

To determine the relative level of apoptosis occurring in Jurkat cells at different H_2O_2 doses, caspase-3 assays were performed. Caspase-3 activity was monitored 6 hr after H_2O_2 exposure, when cells were showing signs of apoptotic membrane blebbing. The Jurkat cells exhibited a sharp increase in caspase-3 activity at the lower doses of H_2O_2, peaking with a 12-fold increase in activity at 50 μM H_2O_2 (Figure 3.7). Higher doses of H_2O_2 (>400 μM) did not activate caspase-3, indeed caspase-3 activity dropped below that seen in untreated cells.

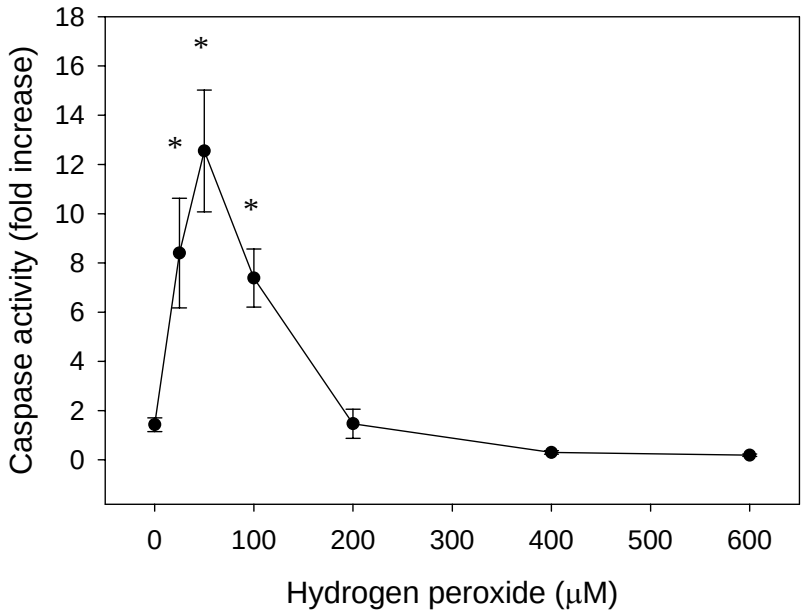


Figure 3.7: Caspase-3 activity in H_2O_2 treated Jurkat cells. *Jurkat cells were exposed to a range of H_2O_2 doses for 6 hr. Cell samples were subsequently removed and assayed for caspase-3 activation by monitoring cleavage of DEVD-AMC. Values represent the mean ± SE of 3 independent experiments. * Indicates a significant difference ($p<0.05$) from untreated cells as determined by a One Way Repeated Measures ANOVA with Bonferroni multiple comparison (SigmaStat).*

3.3.5 Effect of Bcl-2 on H_2O_2-induced cell death

The Bcl-2 transfectants were exposed to a range of H_2O_2 doses in order to determine the effect of Bcl-2 expression on cell death. The Neo-1 clone displayed a similar level of sensitivity to H_2O_2 as that seen in the parental Jurkat cells (Figure 3.8). The Bcl-2 transfectants (B2, B36 and B9) had an elevated level of protection against cell death following H_2O_2 exposure. The B2 clones had an intermediate level of resistance, with an LD_{50} of approximately 100 μM. The B36 and B9 clones were highly resistant to H_2O_2, with an LD_{50} of approximately 200 μM. The difference in cell death seen for the Jurkat and B9 cells treated with 100 μM H_2O_2 was best illustrated by examining the contrast in PI staining, as determined by flow cytometry (Figure 3.8 inset). The data provided a statistically significant correlation ($R^2 = 0.7542$, $P<0.05$) between Bcl-2 expression and resistance towards H_2O_2 (Figure 3.9).

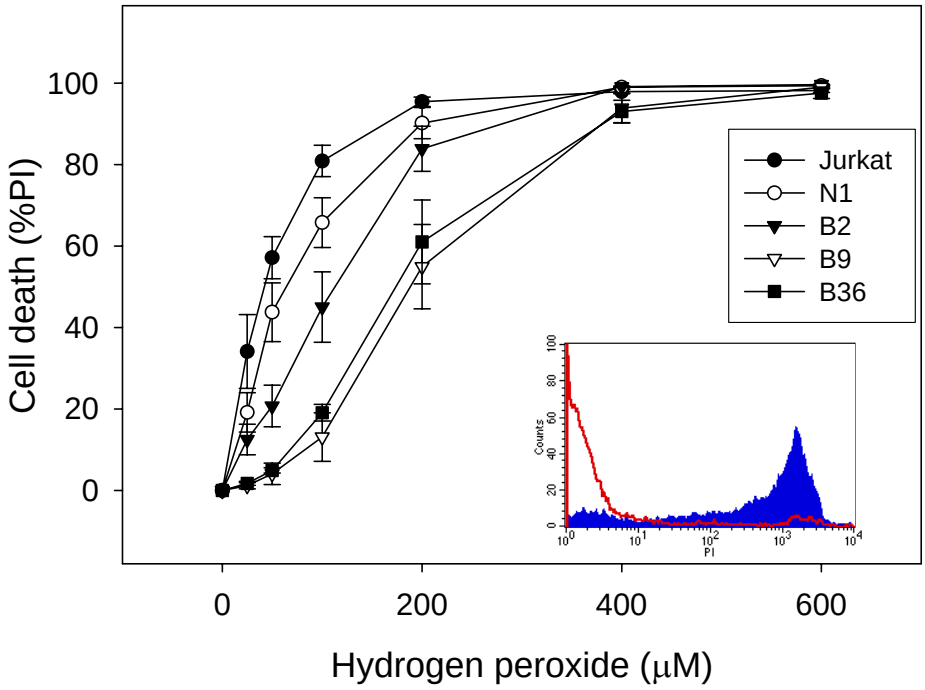


Figure 3.8: Cell viability of Bcl-2 transfectants exposed to H_2O_2 after 24 hr. *The Bcl-2 transfectants and control cells were challenged with a range of H_2O_2 doses and incubated for 24 hr. Cells were subsequently stained with PI and subjected to FACS analysis to differentiate the proportion of PI positive cells in the population. Values represent the mean ± SE of 4 independent experiments. Inset is a FACS flow cytometry histogram of Jurkat (blue) and B9 (overlaid red) cells treated with 100 μM H_2O_2 24 hr after exposure.*

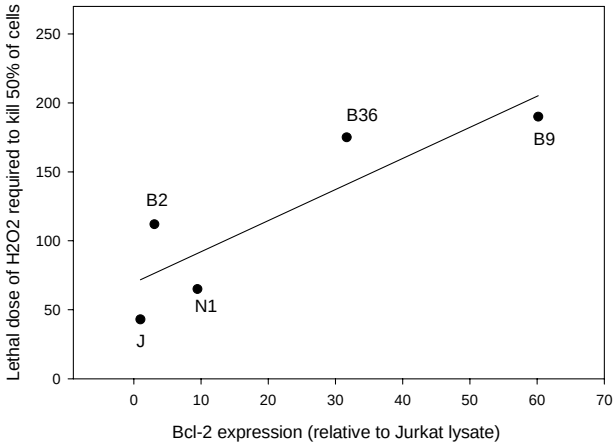


Figure 3.9: Correlation between Bcl-2 expression and the LD_{50} of each clone to H_2O_2. *The lethal dose required to kill 50% of the cells (LD_{50}) in a population was determined from the cell death curves above. The LD_{50} values were plotted against the level of Bcl-2 expression for each clone and a significant correlation (R^2=0.7542 and $p<0.05$) was determined using SigmaStat (Jandel scientific).*

3.3.6 Effect of Bcl-2 on the induction of apoptosis and necrosis

Having shown that Bcl-2 protected Jurkat cells from H_2O_2-induced cell death, it was important to understand whether Bcl-2 was blocking apoptosis, necrosis, or both. The B9 clones exhibited distinct differences in cell morphology when treated with H_2O_2 (Figure 3.10). H_2O_2-treated B9 clones showed no signs of apoptotic cell death at any dose, but did become necrotic at higher doses.

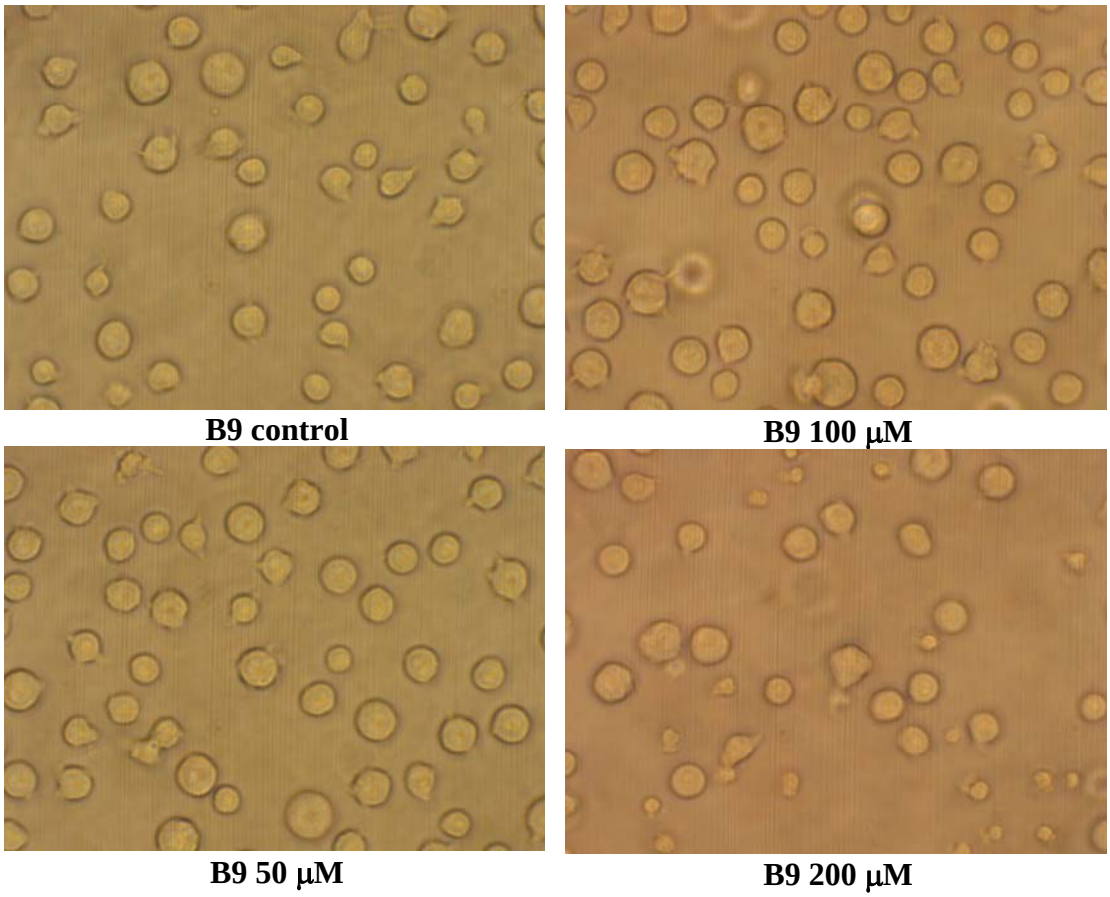


Figure 3.10: Morphology of B9 clones exposed to H_2O_2. *Cells were treated with H_2O_2 for 6 hr. Images are representative of 3 independent experiments.*

These phenotypic observations of cell death were verified by carrying out caspase-3 activity assays. As expected, the Neo-1 clone showed a biphasic response in caspase activity, similar to the parental Jurkat cell line, with caspase-3 activity present at the lower doses of H_2O_2 (Figure 3.11). However, there was no caspase 3 activation in the Bcl-2 transfectants, with the exception of the B2 clones, which peaked with a 3-fold increase in activity at 50 μM H_2O_2. To prove that Bcl-2 was not just delaying

apoptosis, caspase-3 assays were performed at later time-points (data not shown). These data failed to reveal any activation of caspase-3 in the Jurkat cells overexpressing Bcl-2.

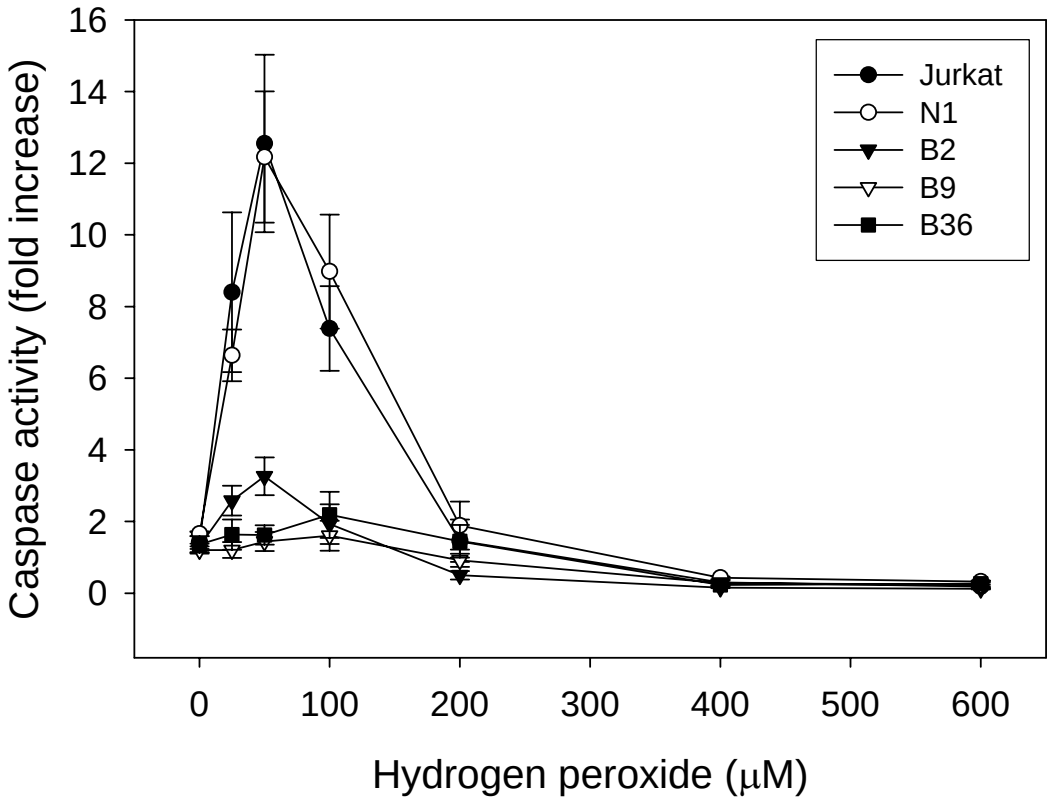
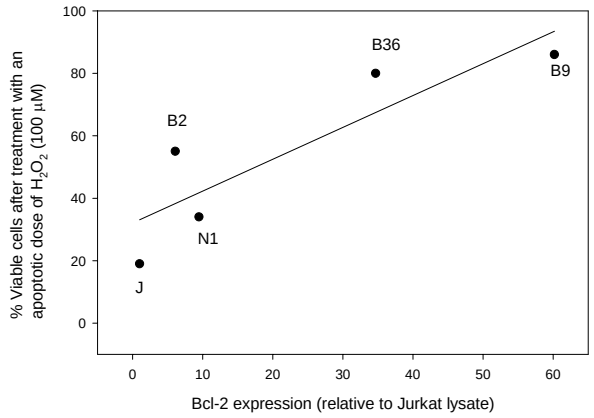


Figure 3.11: Caspase-3 activity in H_2O_2 treated Bcl-2 transfectants. *The Bcl-2 transfectants were exposed to a range of H_2O_2 doses for 6 hr. Cell samples were subsequently removed and assayed for caspase-3 activation by monitoring the cleavage of DEVD-AMC. Values represent the mean $\pm$ SE of 3 independent experiments.*

The results achieved so far suggest that Bcl-2 protects cells from apoptosis in response to H_2O_2. Correlations were undertaken between Bcl-2 expression and cell survival, after treatment with apoptotic and necrotic doses of H_2O_2 (Figure 3.12). As predicted, there was a significant ($p<0.05$) positive correlation between Bcl-2 expression and cell survival after exposure to an apoptotic dose of H_2O_2. However, no such correlation could be found between Bcl-2 expression and cell survival after being challenged with a necrotic dose of H_2O_2.

A)



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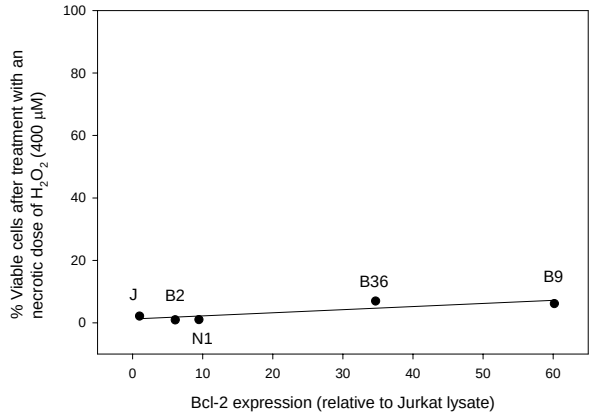


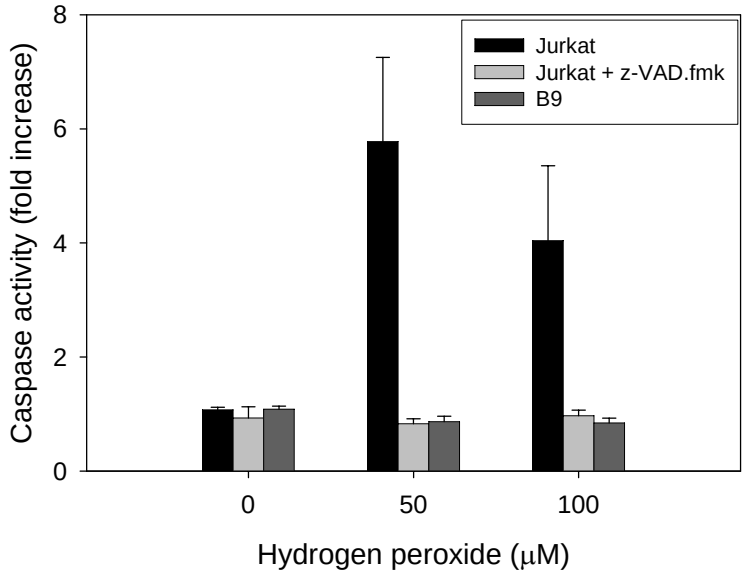
Figure 3.12: Correlation between Bcl-2 expression and cell survival after treatment with apoptotic (A) and necrotic (B) doses of H_2O_2. *A significant correlation was found between Bcl-2 expression and cell survival after exposure to apoptotic doses of H_2O_2 ($p<0.05$), but not necrotic doses of H_2O_2. Values for the percentage of viable cells after treatment were plotted against the level of Bcl-2 expression for each clone and a significant correlation ($R^2=0.7542$ and $p<0.05$) was determined using SigmaStat (Jandel scientific).*

3.3.7 Effect of caspase inhibition on cell viability of H_2O_2 treated Jurkats

The pan-caspase inhibitor z-VAD.fmk was incubated with cells prior to H_2O_2 exposure, in order to differentiate between the resistance provided by caspase inhibition, compared with the protection provided by Bcl-2 overexpression. Jurkat cells incubated with z-VAD.fmk did not undergo the characteristic membrane blebbing in response to H_2O_2. Furthermore, caspase activation was blocked in H_2O_2

treated Jurkats pre-treated with z-VAD.fmk (Figure 3.13A). Caspase inhibition decreased the cell death seen in H_2O_2-treated Jurkat cells to approximately half that of the control (Figure 3.13B). Interestingly, Bcl-2 overexpression provided a far greater level of protection from H_2O_2-induced cell death than caspase inhibition.

A)



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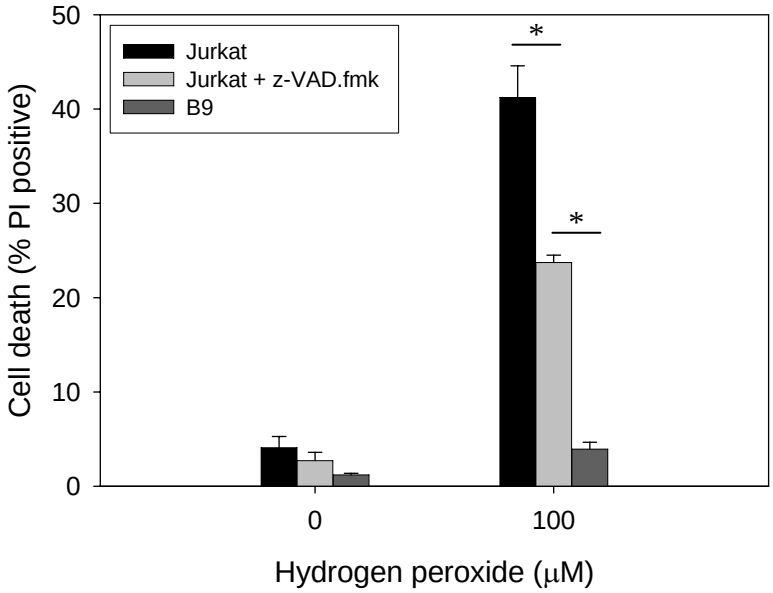


Figure 3.13: Response of Jurkat cells treated with H_2O_2 in the presence of z-VAD.fmk (1 mM). *(A) Cell samples were removed 6 hr after exposure and assayed for caspase-3 activity by monitoring the cleavage of DEVD-AMC. (B) Cells were stained with PI 24 hr after exposure and subjected to FACS analysis to determine the %PI positive (dead) cells. Values represent the mean $\pm$ SE of 3 individual experiments. * Indicates a significant difference ($p<0.05$) between variables as determined by a One Way Repeated Measures ANOVA with Bonferroni multiple comparison (SigmaStat).*

3.4 Discussion

An initial characterisation of the Jurkat cells and Bcl-2 transfectants revealed that Bcl-2 overexpression prevented Fas-mediated apoptosis. Such findings are in agreement with previous studies performed by Kawawhara et al. on Fas-treated Jurkat cells (Kawahara et al. 1998). Treatment of Jurkat cells with H_2O_2 caused a dose-dependent increase in cell death. The LD_{50} ascertained for Jurkat cells treated with H_2O_2 (50 μM) corroborated the findings of former studies (Carayon et al. 1996; Barbouti et al. 2002), and also consistent with previous reports, the cells showed apoptotic morphology at intermediate doses (25-150 μM H_2O_2) and necrotic morphology at higher doses (> 150 μM H_2O_2) (Hampton and Orrenius 1997, 1998; Troyano et al. 2003). This data demonstrated that there was a narrow dose window in which H_2O_2 caused the activation of caspase-3. Hampton et al. have previously investigated the response of Jurkat cells challenged with H_2O_2 (Hampton and Orrenius 1997). In support of our current study, the authors reported a long lag phase before caspase-3 activation (around 4 - 6 hr) and a similar biphasic response in caspase-3 activity.

Bcl-2 protected Jurkat cells from H_2O_2-induced cell death in an expression-dependent manner. The overexpression of Bcl-2 blocked apoptotic features including membrane blebbing and caspase-3 activation. Interestingly, Bcl-2 overexpression only prevented cell death at the doses of H_2O_2 that caused caspase-3 activation (0-125 μM H_2O_2). Hence, Bcl-2 may only be protecting cells from H_2O_2 by blocking the apoptotic response. In support of these findings, a significant positive correlation was found between Bcl-2 expression and cell survival after exposure to apoptotic doses of H_2O_2. However, no such correlation could be established at necrotic doses of H_2O_2. Together, these data are consistent with a model in which Bcl-2 protects cells from H_2O_2 by blocking H_2O_2-induced apoptosis but not necrosis (Figure 3.14).

These findings are consistent with previous studies demonstrating that Bcl-2 blocks oxidant-induced apoptosis, but not necrosis (Torigoe et al. 1994; Ichimiya et al. 1997; Bojes et al. 1998; Ichimiya et al. 1998; Rimpler et al. 1999; Amstad et al. 2001; Jang and Surh 2004). For example, Chen et al. transfected bakers yeast (*Saccharomyces cerevisiae*) with human Bcl-2 and found that the transfectants were protected from

oxidative stress-induced apoptosis (Chen et al. 2003). In the same study, they reported that both Bcl-2 transfected and wild type strains were equally susceptible to verapamil, an inducer of necrosis. However, there are contradictory reports suggesting that Bcl-2 may block necrotic cell death in some circumstances (Kane et al. 1995; Tsujimoto et al. 1997; Troyano et al. 2003). In each case, the death-inducing stimuli are ROS. These findings are consistent with the hypothesis that Bcl-2 may protect cells from oxidants by a mechanism distinct from preventing apoptosis

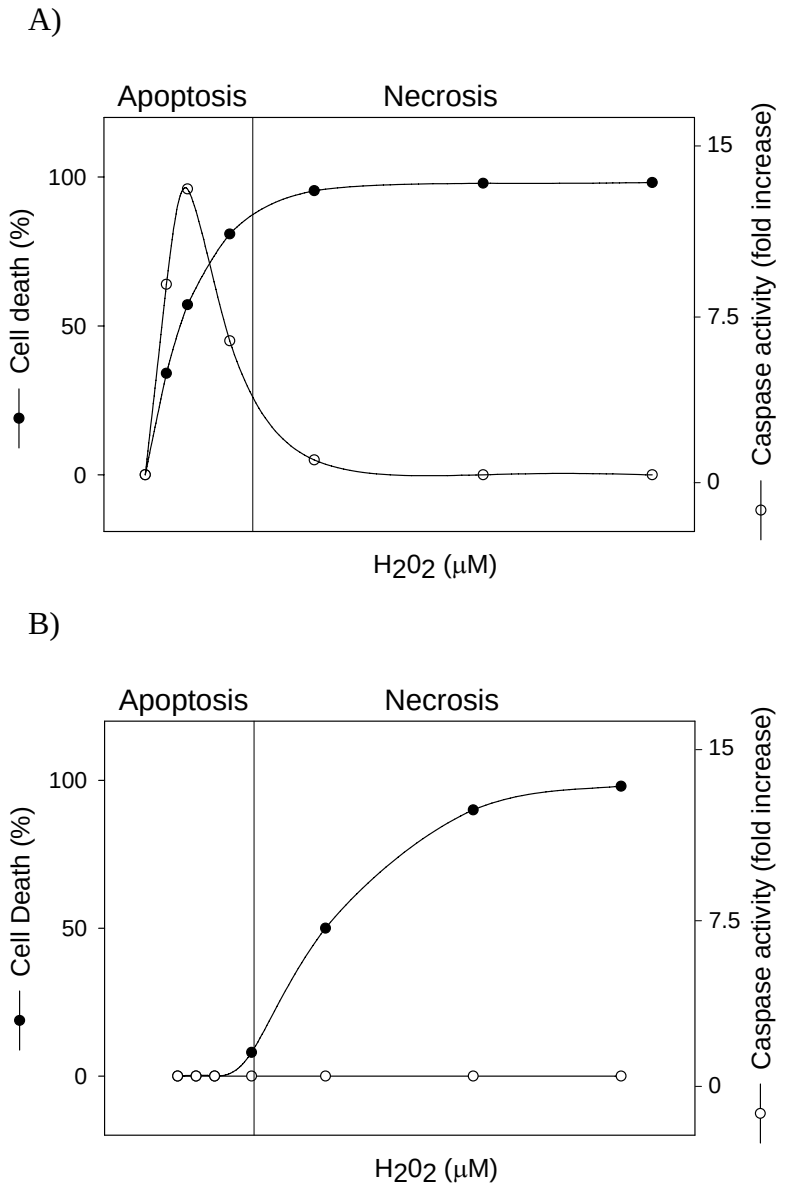


Figure 3.14: Summary model of Jurkat (A) and B9 (B) cells response to H_2O_2. *Cell death (closed circles) and caspase-3 activity (open circles) are modulated depending on the dose. Bcl-2 blocks H_2O_2-induced apoptosis but not necrosis.*

Recent studies have observed that ROS can modulate the expression of Bcl-2 with antioxidants enhancing Bcl-2 expression and ROS downregulating expression (Hildeman et al. 2003; Hildeman 2004). This phenomenon has been observed in Jurkat cells, which downregulate Bcl-2 expression when exposed to 100 μM H_2O_2 (Chiaramonte et al. 2001). Furthermore, ROS can modulate Bcl-2 at the post-translational level by promoting the ubiquitination and subsequent inactivation of Bcl-2 (Li et al. 2004). The modulation of Bcl-2 expression and activity may play a role in the current study, by shifting the apoptosis-survival equilibrium in favour of apoptosis.

Pre-treatment of Jurkat cells with the pan-caspase inhibitor z-VAD.fmk prevented half of the cell death seen in response to 100 μM H_2O_2. The inability of z-VAD.fmk to completely inhibit cell death induced by ROS has been observed in previous studies (Kim et al. 2000; Uedaa et al. 2001; Scoltock and Cidlowski 2004; Scheller et al. 2006). In many cases, z-VAD.fmk simply changes the mode of cell death from apoptosis to necrosis. In contrast, the overexpression of Bcl-2 provided almost complete protection for Jurkat cells exposed to 100 μM H_2O_2. Such findings suggest that Bcl-2 protects cells from H_2O_2 by a mechanism that is distinct from its ability to prevent caspase activation (Figure 3.15).

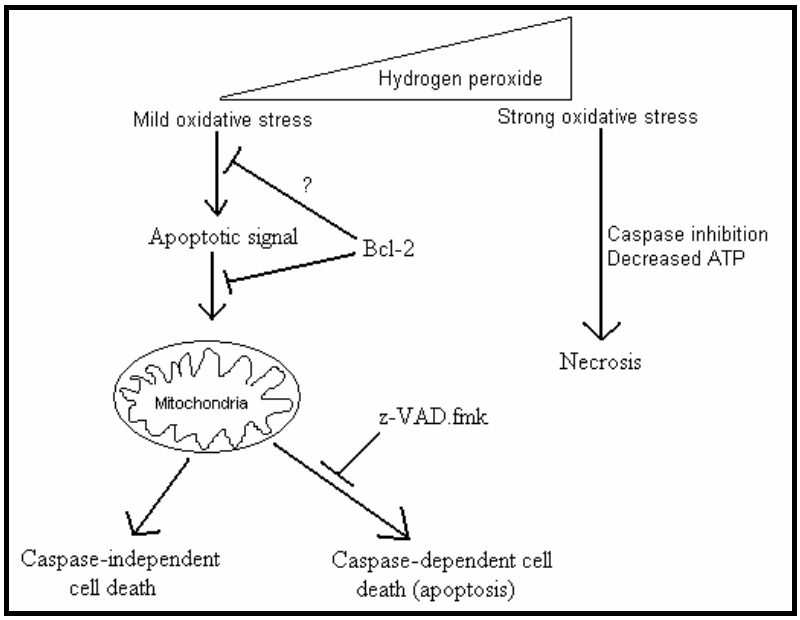


Figure 3.15: Summary diagram showing the pathways of H_2O_2-induced cell death.

Caspase inhibition may not protect cells as well as Bcl-2 overexpression if some form of caspase-independent apoptosis occurs. Alternatively, Bcl-2 may offer additional protection by enhancing the antioxidant status of the cell, and from offering some protection against necrosis that might occur at lower doses of H_2O_2.

In summary, the results in this chapter have shown that Bcl-2 protects cells from H_2O_2-induced apoptosis. The protection provided by Bcl-2 could not be explained by caspase inhibition alone. Hence, it was important to investigate the possible antioxidant properties of Bcl-2 function. The effect of Bcl-2 on the antioxidant capacity of Jurkat cells is the focus of the next chapter.

Chapter 4: Effect of Bcl-2 expression on the antioxidant capacity of Jurkat T cells

4.1 Introduction

Despite a wealth of data, the exact mechanism by which Bcl-2 functions remains controversial. As discussed in chapter 1 (section 1.2.3), several lines of evidence have revealed that Bcl-2 overexpression enhances the antioxidant capacity of the cell (Voehringer 1999; Jang and Surh 2003; Kowaltowski and Fiskum 2005) and it is believed by some researchers that the antioxidant properties of Bcl-2 protect cells from apoptosis. Previous studies have reported a link between Bcl-2 expression and the level of endogenous glutathione (Ellerby et al. 1996; Mirkovic et al. 1997; Meredith et al. 1998; Papadopoulos et al. 1998; Tyurin et al. 1998; Ellerby and Bredesen 2000; Lee et al. 2001; Hoetelmans et al. 2003; Jang and Surh 2003, 2004a). The Bcl-2-dependent rise in glutathione causes a concomitant increase in the concentration of protein thiols (Ellerby et al. 1996; Tyurin et al. 1998). It is well established that glutathione can block the induction of apoptosis by scavenging reactive oxygen species (ROS) (Sies 1999). Interestingly, there are several reports showing that Bcl-2 overexpressing cells can be resensitised to apoptosis by glutathione depletion (Mirkovic et al. 1997; Armstrong and Jones 2002). In addition to glutathione, the overexpression of Bcl-2 has been shown to increase the activity of the major antioxidant enzymes superoxide dismutase (Steinman 1995; Ellerby et al. 1996; Bruce-Keller et al. 1998; Papadopoulos et al. 1998; Lee et al. 2001), catalase (Steinman 1995; Bruce-Keller et al. 1998; Del Bufalo et al. 2001; Lee et al. 2001; Jang and Surh 2004b), and glutathione peroxidase (Bruce-Keller et al. 1998; Papadopoulos et al. 1998). The overexpression of these antioxidant enzymes has been shown to protect cells from apoptosis (Sandstrom and Buttke 1994; Rabizadeh et al. 1995; Gouaze et al. 2002). Other antioxidants, such as the peroxiredoxins, can also block apoptosis by preventing the accumulation of ROS (Zhang et al. 1997; Chang et al. 2004). As yet, no antioxidant enzyme has consistently been shown to be upregulated in cells overexpressing Bcl-2, suggesting that the impact of Bcl-2 on the activity of antioxidant enzymes is cell line-dependent. Indeed, several studies have reported that Bcl-2 has no

effect on the activity of antioxidant enzymes (Lee and Shacter 1997; Schor et al. 2000; Amstad et al. 2001).

In the previous chapter, it was demonstrated that Bcl-2 could protect cells from apoptosis at intermediate doses of H_2O_2. This could be due to its broad-spectrum inhibition of apoptosis, or the ability to protect the cells against oxidative stress. The purpose of the current chapter was to test the second possibility by performing a systematic study of the effect of Bcl-2 overexpression on the antioxidant status of Jurkat cells.

The main objectives of this chapter were:

1) To determine whether the levels and activity of antioxidant enzymes were altered in Jurkat cells overexpressing Bcl-2.

2) To examine the link between Bcl-2 expression and cellular thiol homeostasis in Jurkat cells overexpressing Bcl-2.

4.2 Experimental approach

Jurkat cells and Bcl-2 transfectants, as described in chapter 3 (section 3.2), were cultured and cell lysates prepared. The lysates were tested for antioxidant enzyme expression and activity. Superoxide dismutase activity was measured by employing a superoxide ($O_2^{-\cdot}$) generating system (xanthine/xanthine oxidase) to reduce a water-soluble tetrazolium dye (WST-1) in the presence of a range of concentrations of superoxide dismutase (Peskin and Winterborn 2000). The presence of superoxide dismutase in the reaction mixture removes $O_2^{-\cdot}$ and thereby prevents the reduction of WST-1. Catalase activity was determined by measuring the decomposition of H_2O_2 over time on the spectrophotometer (Aebi 1984). A coupled spectrophotometer assay involving glutathione reductase was utilised to monitor glutathione peroxidase activity (Flohe and Gunzler 1984). Thioredoxin reductase activity was determined by measuring the NADPH-dependent reduction of the thiol-reactive probe 5,5'-dithiobis (2-nitrobenoic acid) (DTNB) (Arner et al. 1999). The expression level of the antioxidant proteins glutathione peroxidase (1, 3 and 4), thioredoxin reductase (1 and 2), thioredoxin 1 and the peroxiredoxins (1,2,3,4,5

and 6) were determined by immunoblotting. Intracellular thiols were measured using DTNB (Ellman 1958). 5-Iodoacetamidofluorescein (5-IAF) was utilised to specifically label protein thiols, which were subsequently visualised by electrophoresis (Baty et al. 2002). H_2O_2 consumption was measured by using the ferrous oxidation of xylenol orange (FOX) assay (Wolff 1994).

4.3 Results

4.3.1 Assessing superoxide dismutase activity in Jurkat cells and Bcl-2 transfectants

Superoxide dismutase (SOD) is an antioxidant enzyme that converts $O_2^{-\cdot}$ to H_2O_2 (Fridovich 1995). Two isoforms of SOD, CuZn-SOD and Mn-SOD, are present in cells. While CuZn-SOD is localised to the cytoplasm, Mn-SOD is present in the mitochondria (MacMillan-Crow and Cruthirds 2001; McCord 2002). The total SOD activity of each lysate was examined by monitoring the inhibition of WST-1 reduction in the presence of a range of cell lysate concentrations (Figure 4.1). SOD activity was calculated by determining the IC_{50} for each lysate, which, by definition, is equal to one unit of SOD activity (Peskin and Winterborn 2000).

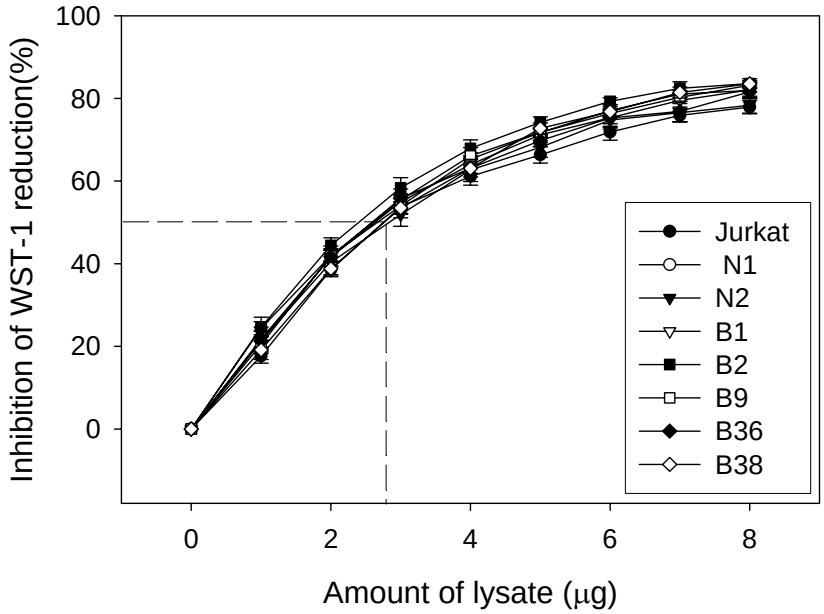
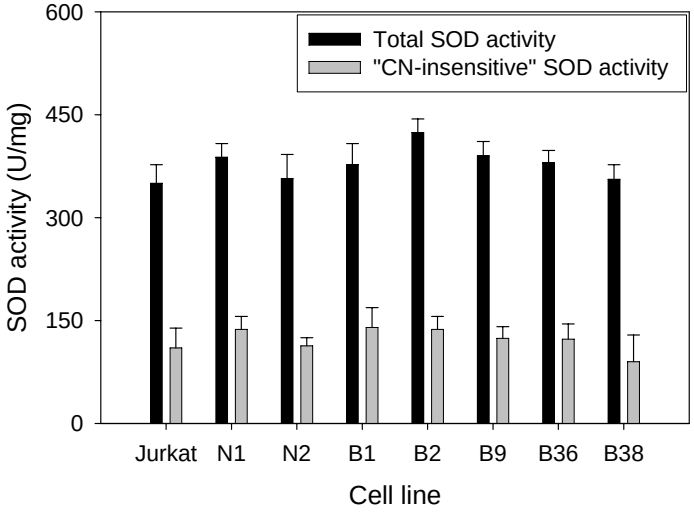


Figure 4.1: Determination of SOD activity. *SOD activity (U/mg) was calculated by estimating the amount of lysate required to inhibit WST-1 reduction by 50% (IC_{50}).*

The cell lysates displayed a similar level of SOD activity, ranging from 350-424 U/mg (Figure 4.2A). There was no significant correlation between Bcl-2 and total SOD activity (P>0.05) (Figure 4.2B). The SOD assay was also performed in the presence of 5 mM KCN, which inhibits CuZn-SOD, leaving only active Mn-SOD (Paci et al. 1988). The "CN-insensitive" SOD (Mn-SOD) activity was very similar in each of the Bcl-2 transfectants, ranging from 100-130 U/mg (Figure 4.2A). Again, there was no significant correlation between Bcl-2 expression and the activity of "CN-insensitive" SOD (Figure 4.2B).

A)



B)

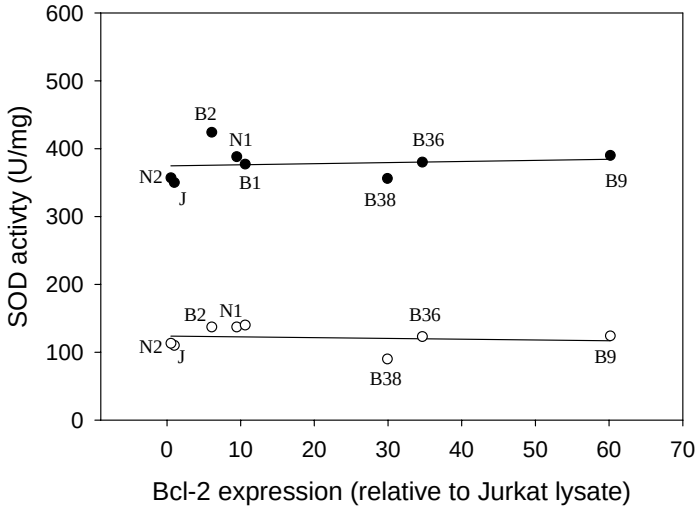
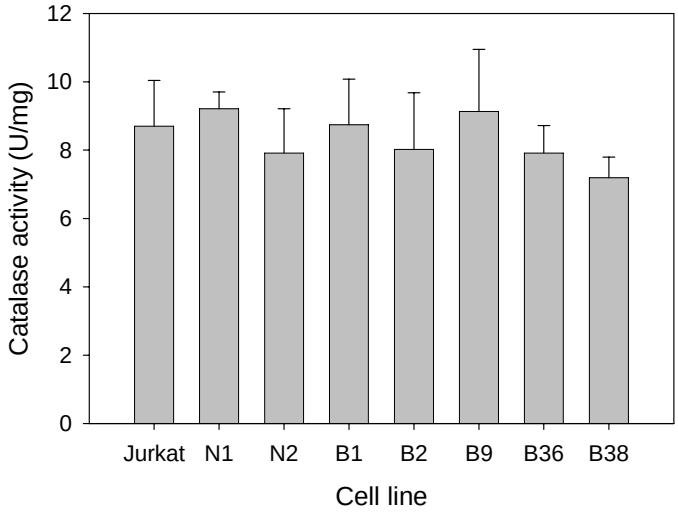


Figure 4.2: SOD activity in cell lysates of Bcl-2 transfectants. *Values represent the mean ± SE of 3 independent experiments. "CN-insensitive" SOD activity was monitored in the presence of 5 mM KCN. Correlation between Bcl-2 expression and SOD activity generated using Sigma stat (not significant P>0.05).*

4.3.2 Assessing catalase activity in Jurkat cells and Bcl-2 transfectants

Catalase is an antioxidant enzyme that decomposes H_2O_2 to form oxygen and water (Aebi 1984; Brioukhanov and Netrusov 2004). In order to evaluate whether Bcl-2 expression affects catalase function, cell lysates of the Bcl-2 transfectants were assayed for catalase activity. Catalase activity showed little variability between the clones, ranging from 7.1-9.2 U/mg (Figure 4.3A). Moreover, there was no correlation between the activity of catalase and the level of Bcl-2 expression (P>0.05) (Figure 4.3B).

A)



B)

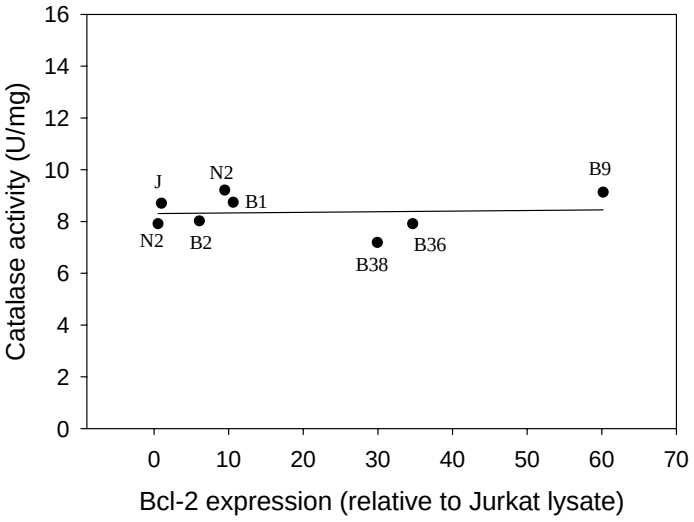
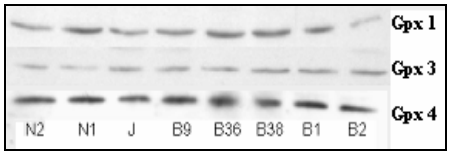


Figure 4.3: Catalase activity in cell lysates of Bcl-2 transfectants. *Catalase activity was measured in cell lysates by following the decomposition of H_2O_2 on a spectrophotometer at 240nm. Values represent the mean $\pm$ SE of 3 independent experiments. B) Correlation between Bcl-2 expression and catalase activity generated using Sigma stat (not significant P>0.05).*

4.3.3 Assessing glutathione peroxidase in Jurkat cells and Bcl-2 transfectants

The glutathione peroxidases are ubiquitous antioxidant enzymes that use glutathione to decompose H_2O_2 (Lei 2002). There are five isoforms that reside in different sub-cellular locations, one of which (glutathione peroxidase 4) specifically reduces organic hydroperoxides (Imai and Nakagawa 2003). To clarify the influence of Bcl-2 expression on glutathione peroxidase, both the level of expression and activity of the enzyme were determined. The expression of glutathione peroxidase 1, 3, and 4 was monitored via a densitometry analysis of the immunoblot band of interest, which travelled at approximately 26 kDa (Figure 4.4). The densitometry analysis revealed that there was no significant correlation ($P>0.05$) between Bcl-2 expression and the expression of any of the glutathione peroxidase isoforms (Figure 4.5). The glutathione peroxidase activity showed some variation among the Bcl-2 transfectants ranging from 0.093-0.13 U/mg (Figure 4.6A); however, a lack of correlation between Bcl-2 expression and glutathione peroxidase activity demonstrated that there was no statistically significant trend between the variables ($P>0.05$) (Figure 4.6B).

A)



B)

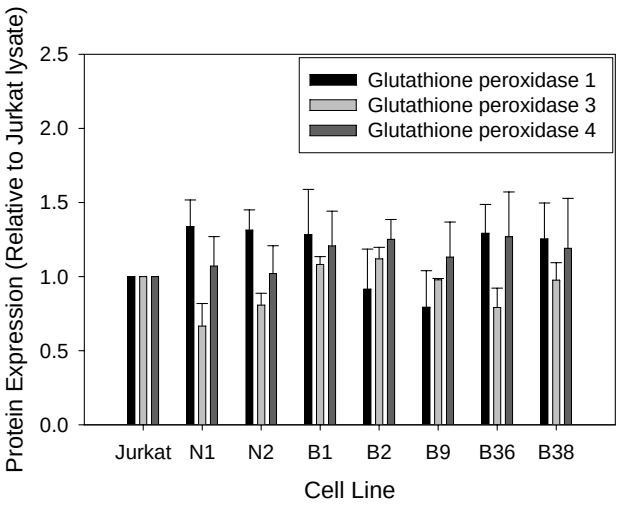
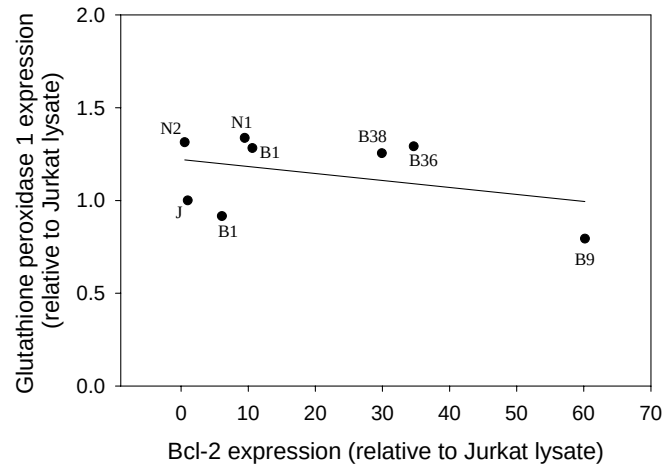


Figure 4.4: Immunoblot detection of the glutathione peroxidases (isoforms 1, 3 and 4). *(A) Cell lysates were resolved by SDS-PAGE and immunoblotted using polyclonal antibodies against Glutathione peroxidase (Gpx) 1, 3 and 4 respectively. (B) Densitometry histogram of immunoblots in the Bcl-2 transfectants. The bands were quantified using Quantity-One (BioRad). Values represent the mean ± SE of 3 independent experiments.*

A)



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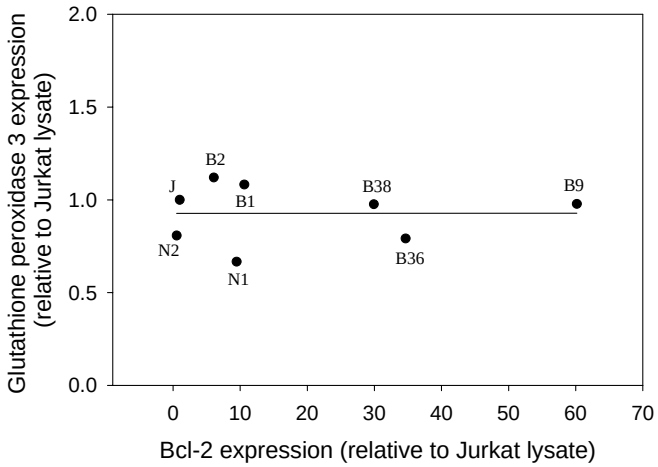

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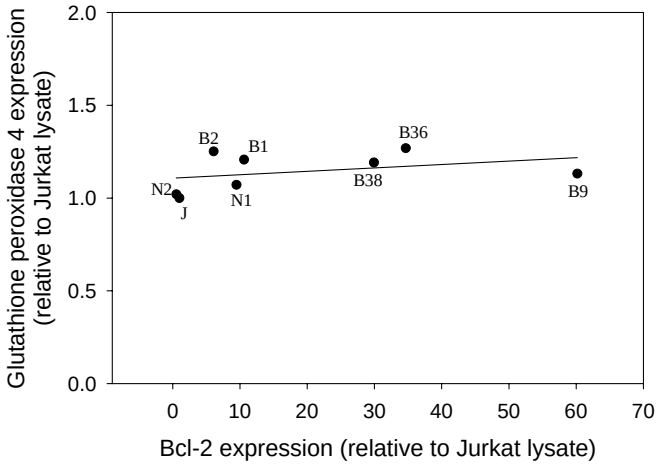
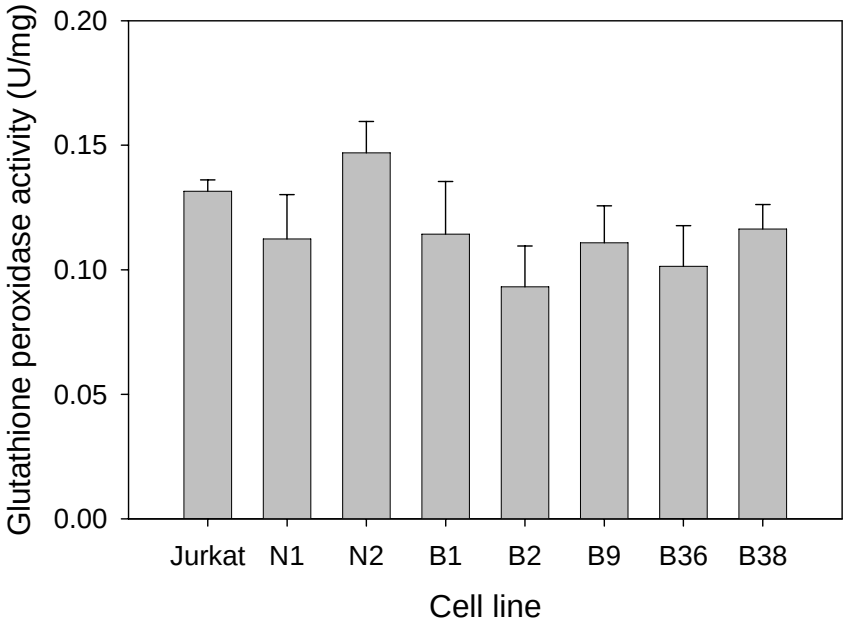


Figure 4.5: Correlations between Bcl-2 expression and glutathione peroxidase expression. *Correlations were generated using Sigma stat (not significant P>0.05). Values were determined by immunoblot detection (Figure 4.4) and represent the mean of at least 3 independent experiments.*

A)



B)

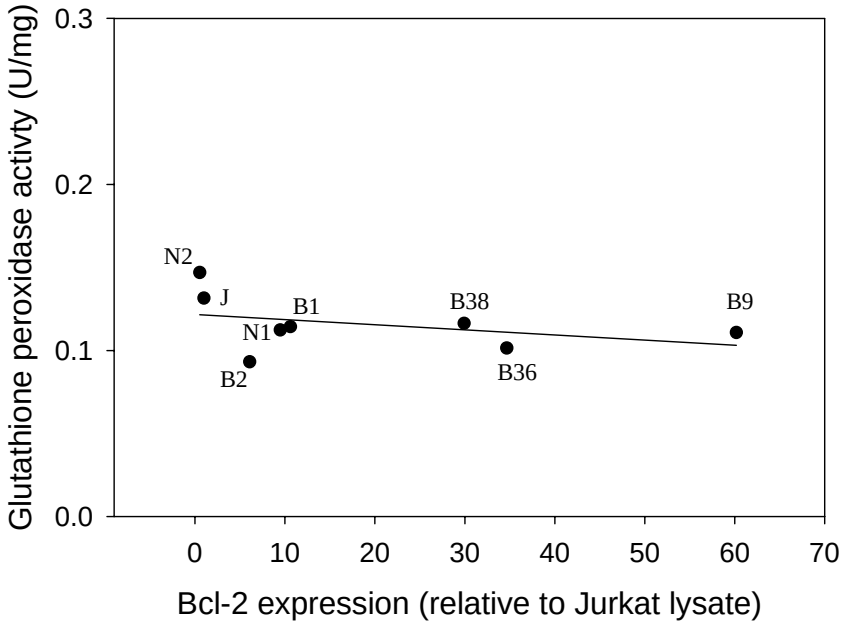


Figure 4.6: Glutathione peroxidase activity in cell lysates of Bcl-2 transfectants. *(A) Cell lysates were assayed for glutathione peroxidase activity in a spectrophotometer assay developed by Flohe et el (Flohe and Gunzler 1984). Values represent the mean ± SE of 3 independent experiments. (B) Correlation between Bcl-2 expression and glutathione peroxidase activity generated using Sigma stat (not significant P>0.05).*

4.3.4 Assessing the levels of the peroxiredoxin isoforms in Bcl-2 transfectants

The peroxiredoxins are a novel family of peroxidases that use a redox-active cysteine to decompose H_2O_2 (Rhee et al. 2005b). The six mammalian peroxiredoxin isoforms reside in different cellular compartments and use different mechanisms to reduce H_2O_2 (Wood et al. 2003). The expression levels of the peroxiredoxins (isoforms 1-6) were tested in the Bcl-2 transfectants using isoform-specific antibodies (Figure 4.7). A densitometry analysis of the Western blots revealed that there were no significant differences in the expression of any peroxiredoxin in any of the Bcl-2 transfectants (Figure 4.8). Furthermore, correlations between Bcl-2 expression and peroxiredoxin expression were not significant ($P>0.05$).

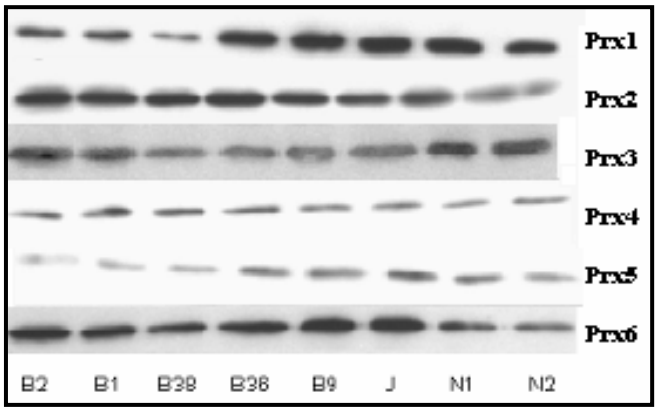
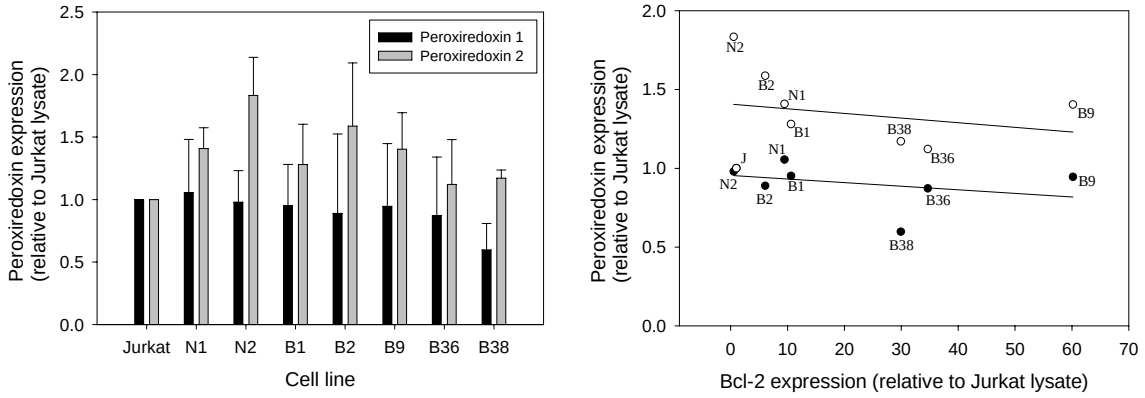


Figure 4.7: Immunoblot detection of the peroxiredoxins (isoforms 1-6). *Cell lysates were resolved by SDS-PAGE and immunoblotted using polyclonal antibodies against peroxiredoxin 1-6 (Prx 1-6) respectively. Western blots are representative of at least 3 independent experiments.*

A)



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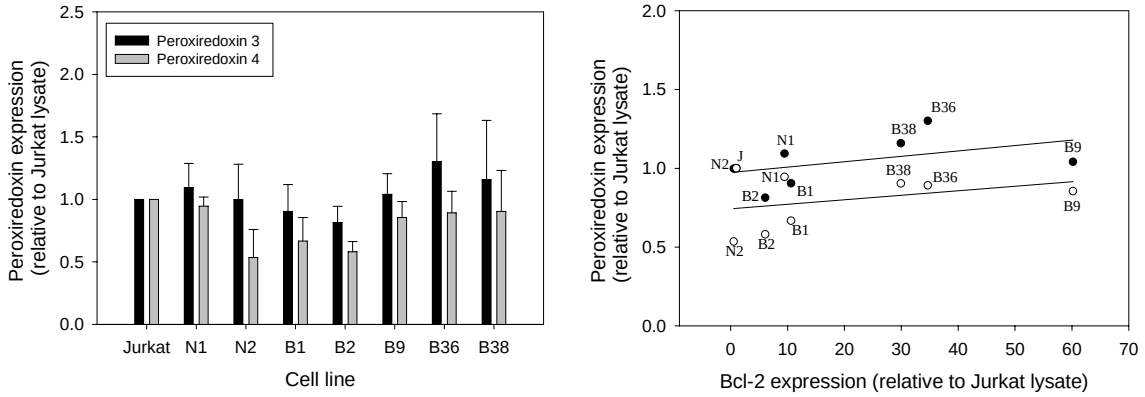

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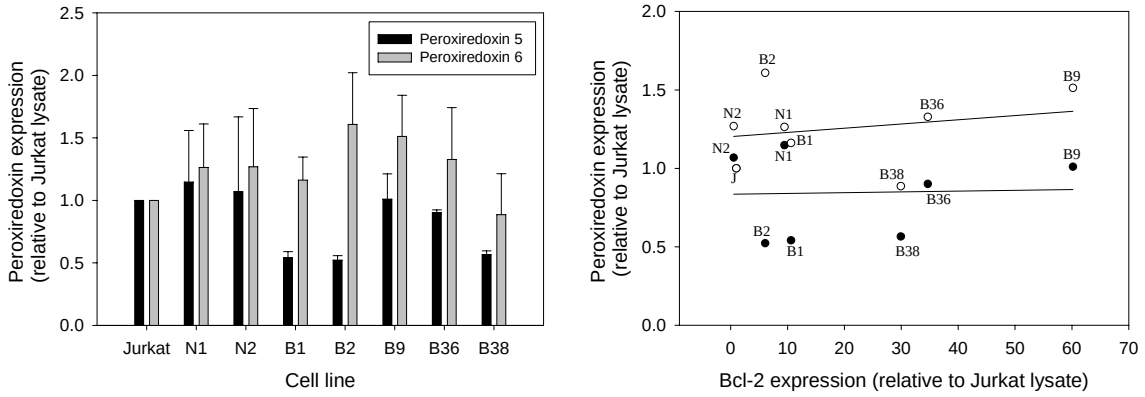
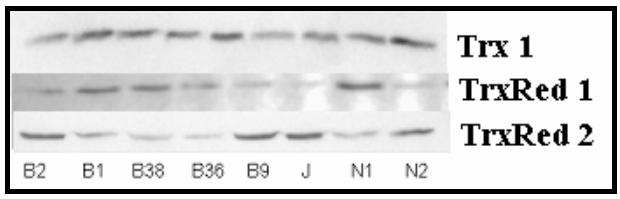


Figure 4.8: Peroxiredoxin expression in Bcl-2 transfectants. *Densitometry analysis of peroxiredoxins 1-2 (A), 3-4 (B) and 5-6 (C) for each cell line. The bands were quantified using Quantity-One (BioRad). Values represent the mean ± SE of 3 independent experiments. Correlations between Bcl-2 expression and peroxiredoxin expression were determined using Sigma stat. Closed circles represent peroxiredoxin 1 (A), 3(B) and 5 (C) while open circles represent peroxiredoxin 2 (A), 4 (B) and 6(C). No correlations reached significance (not significant P>0.05).*

4.3.5 Assessing the thioredoxin/thioredoxin reductase system in Bcl-2 transfectants

Given that Bcl-2 has been reported to augment the redox potential of a cell, the thioredoxin/thioredoxin reductase system in the Bcl-2 transfectants was assessed. The thioredoxin/thioredoxin reductase system is involved in reducing oxidised protein thiols and disulfides at the expense of NADPH (Mustacich and Powis 2000). The levels of thioredoxin 1, thioredoxin reductase 1, and thioredoxin reductase 2 were detected by immunoblotting and densitometry (Figure 4.9). The densitometry revealed no significant changes in the levels of thioredoxin 1, thioredoxin reductase 1 or 2 in the Bcl-2 transfectants. Furthermore, there was no significant correlation between Bcl-2 expression and the level of thioredoxin or thioredoxin reductase 1 and 2 ($P>0.05$) (Figure 4.10).

A)



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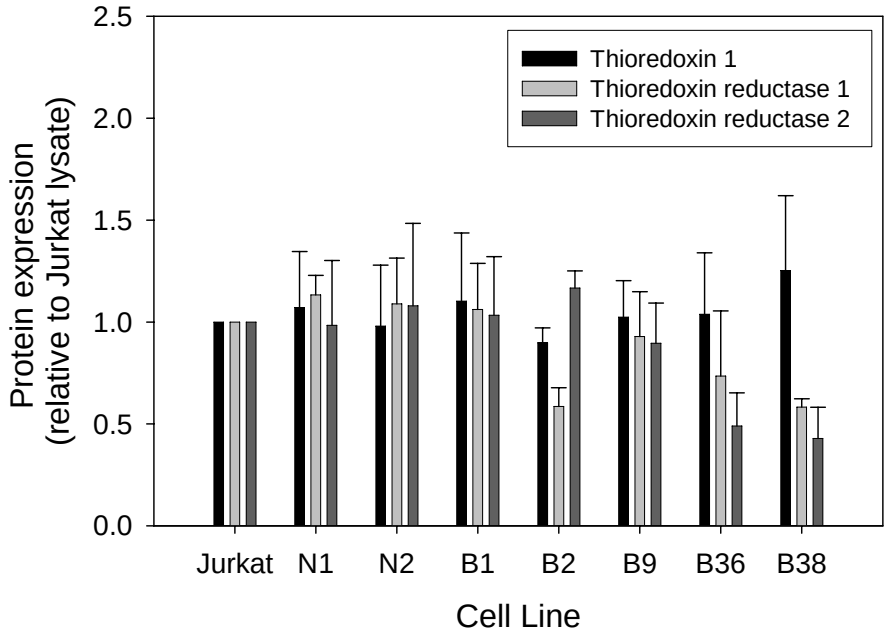
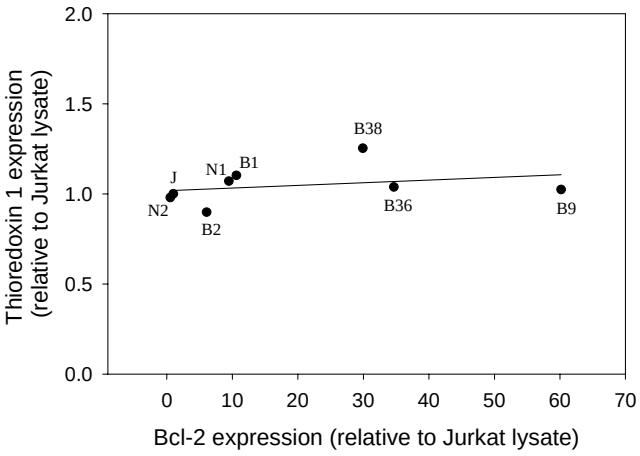


Figure 4.9: Immunoblot detection of proteins involved in the thioredoxin reducing system. *Cell lysates were resolved by SDS-PAGE and immunoblotted using polyclonal antibodies against thioredoxin 1 (Trx1) thioredoxin reductase 1 (TrxRed1) and thioredoxin reductase 2 (TrxRed 2) respectively (previous page, A). B) Densitometry analysis of immunoblots. Bands were quantified using Quantity-One (BioRad). Values represent the mean ± SE of 3 independent experiments.*

A)



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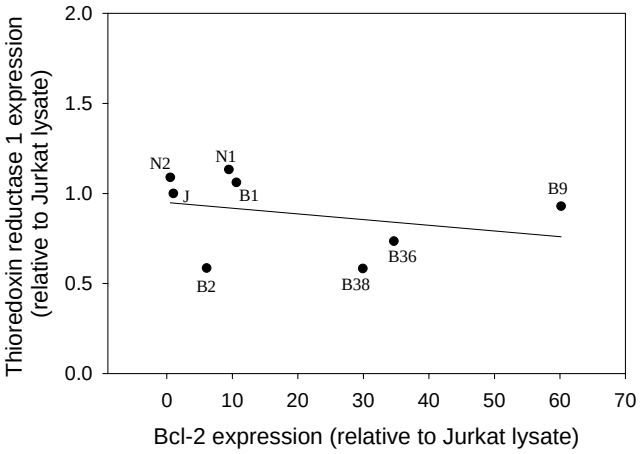

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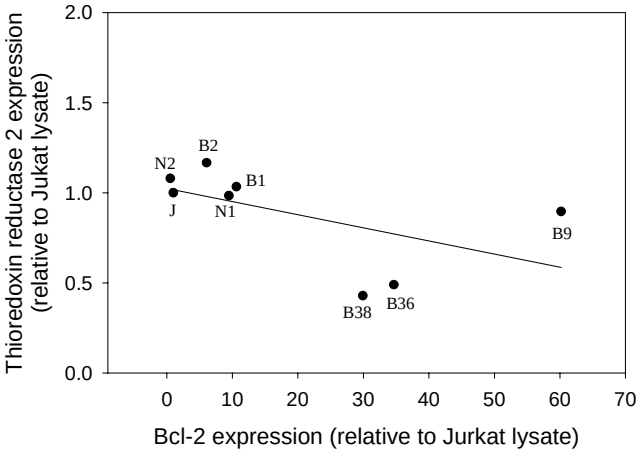


Figure 4.10: Correlation between Bcl-2 and thioredoxin or thioredoxin reductase expression. *Correlations between Bcl-2 expression and Trx 1 (A), TrxRed 1 (B), or TrxRed 2 (C) expression were generated using Sigma stat (not significant P>0.05).*

In addition to immunoblotting, the activity of thioredoxin reductase was ascertained for each Bcl-2 transfectant. The activity of thioredoxin reductase did not vary much between the transfectants ranging from 0.072-0.095 ΔA_{412} nm/min (Figure 4.11A). Moreover, no significant correlation was found between Bcl-2 expression and thioredoxin reductase activity (P>0.05) (Figure 4.11B).

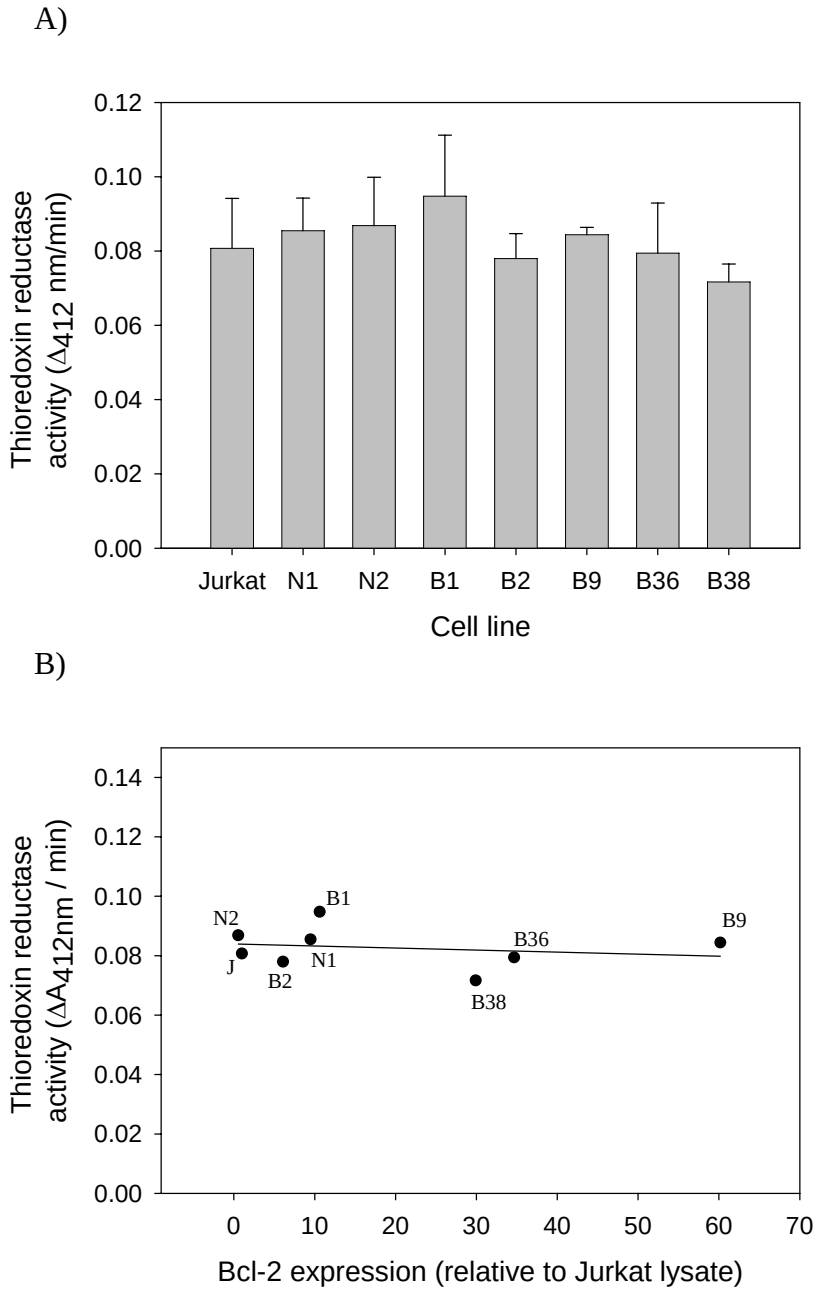
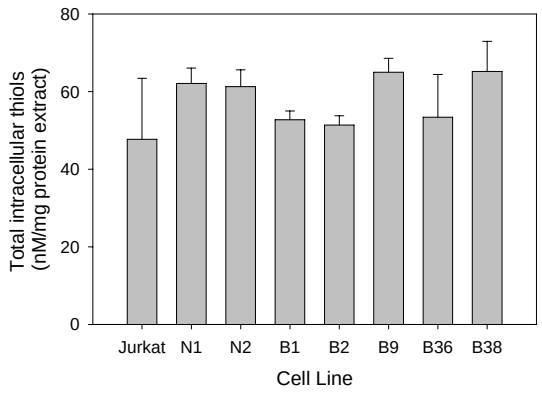


Figure 4.11: Activity of thioredoxin reductase in cell lysates of Bcl-2 transfectants. *(A) Cell lysates were assayed for thioredoxin reductase activity by measuring the NADPH-dependent reduction of DTNB. Values represent the mean ± SE of 3 independent experiments. (B) Correlation between Bcl-2 expression and thioredoxin reductase activity generated using Sigma stat (not significant P>0.05).*

4.3.6 Determining the level of endogenous thiols present in Bcl-2 transfectants

Endogenous thiols can act as cellular antioxidants by scavenging ROS. The total concentration of endogenous thiols, which includes both low molecular weight thiols and protein thiols, was examined in cell lysates of the Jurkat cells and Bcl-2 transfectants. The level of endogenous thiols was relatively similar among the cells tested, ranging from 50-70 nM/mg (Figure 4.12A). To determine whether or not there was any tendency for Bcl-2 overexpressing cells to have more intracellular thiols, a correlation between Bcl-2 expression and intracellular thiol concentration was performed (Figure 4.12B). The data confirmed that there was no statistically significant correlation between Bcl-2 expression and intracellular thiol concentration ($p>0.05$).

A)



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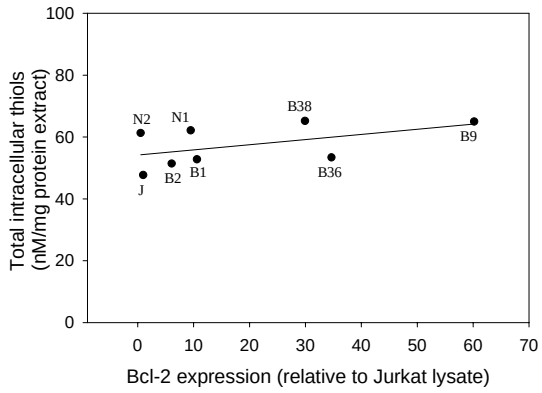
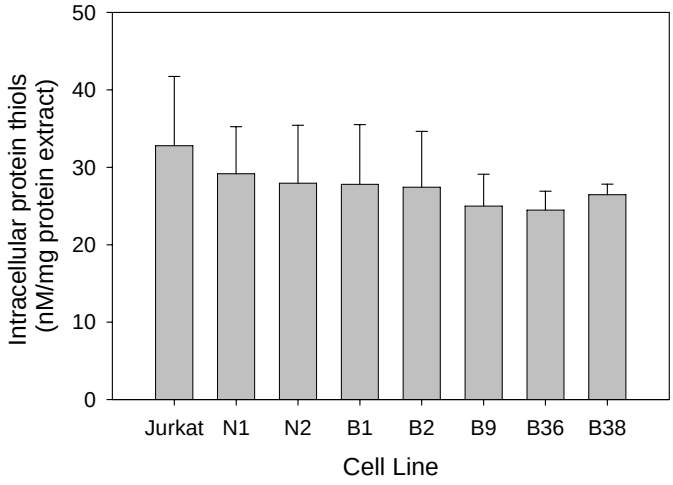


Figure 4.12: Detection of reduced thiols in cell extracts using the DTNB assay. *(A) Cell extracts were aliquoted into a microplate and reacted with DTNB to produce a yellow product (TNB) that could be detected by spectrophotometry at 412 nm. Sample readings were compared to a standard curve of glutathione to generate thiol concentrations (nM/mg) for each cell lysates. Values represent the mean $\pm$ SE of 3 independent experiments. (B) Correlation between Bcl-2 expression and intracellular thiols generated using Sigma stat (not significant P>0.05).*

Protein thiols were isolated from cell lysates by TCA precipitation. The isolation of protein thiols ensured that low molecular weight redox buffering molecules, such as glutathione, would be excluded. The protein thiol concentrations in the Bcl-2 transfectants ranged from 25-32 nM/mg (Figure 4.13A). Interestingly, there was a negative correlation between Bcl-2 expression and protein thiol concentration that just reached significance (P<0.05) (Figure 4.13B).

A)



B)

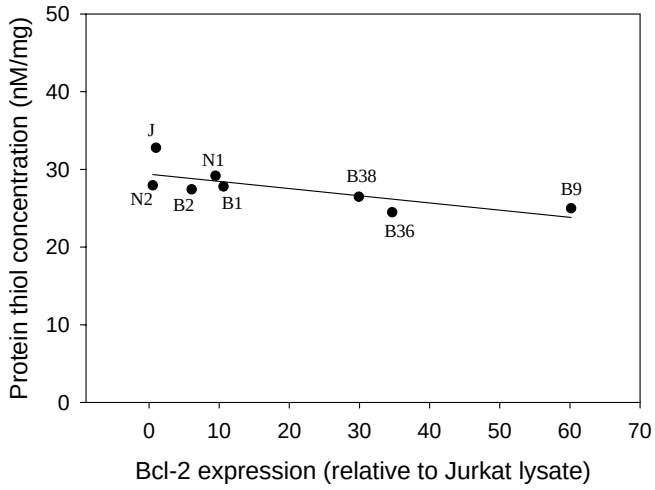


Figure 4.13: Detection of reduced protein thiols in cell extracts using the DTNB assay. *(A) Protein was extracted from cell extracts by TCA precipitation. Extracted protein was resolubilised, reacted with DTNB and detected by spectrophotometry at 412 nm. Thiol concentrations (nM/mg) were generated using a standard curve of glutathione. Values represent the mean ± SE of 3 independent experiments. (B) Significant negative correlation between Bcl-2 expression and protein thiols generated using Sigma stat (P< 0.05, r^2 = -0.715).*

In order to further investigate changes in protein thiols between the Bcl-2 transfectants, an alternative method of protein thiol labelling was utilised. Protein thiols from cell lysates were labelled using the thiol-reactive fluorescent probe 5-IAF. 5-IAF labelled cell lysates from Bcl-2 transfectants were analysed by SDS-PAGE and imaged using a BioRad FX fluorescence scanner. Despite slight variations in band intensity, due to protein loading, there were no major differences in the banding pattern of each cell line (Figure 4.14).

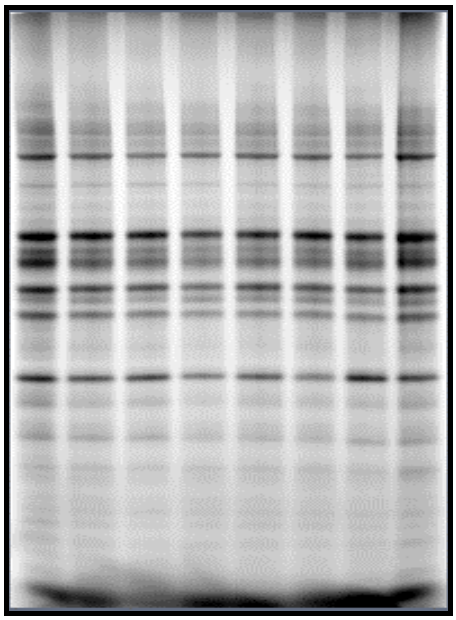


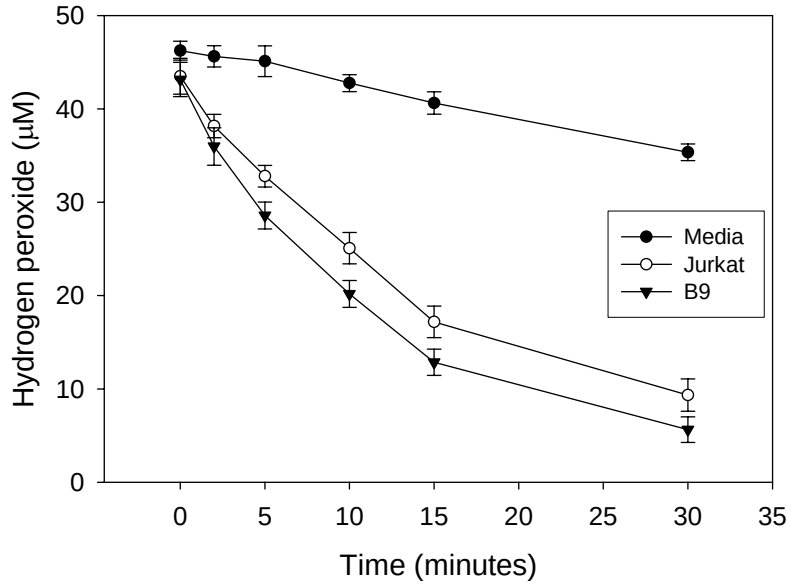
Figure 4.14: Illustrative gel of IAF-labelled reduced thiols in cell extracts. *The gel is illustrative of 3 independent experiments. Cell extracts from the Bcl-2 transfectants were incubated with 200 μM 5-IAF and resolved by SDS-PAGE. Gels were imaged with a BioRad FX fluorescence scanner using Ex λ=488 nm and Em λ=530 nm (Bio-Rad Laboratories, Hercules, CA, USA).*

4.3.7 Assessing the rate of H_2O_2 consumption in Jurkat and B9 cells

H_2O_2 consumption was measured in Jurkat and B9 cells overexpressing Bcl-2 following treatment with a 50 μM or 200 μM bolus of H_2O_2. The FOX assay data revealed that the Jurkat and B9 cells consumed the 50 μM bolus of H_2O_2 at a comparable rate (Figure 4.15A). The B9 cells consumed more H_2O_2 during the initial 2 minutes of exposure; however, this was not statistically significant. A similar trend for H_2O_2 consumption was

seen in Jurkat and B9 cells treated with 200 μM H_2O_2 (Figure 4.15B). Interestingly, the majority of the 200 μM H_2O_2 dose was consumed within the 30-minute time course in both cell lines.

A)



B)

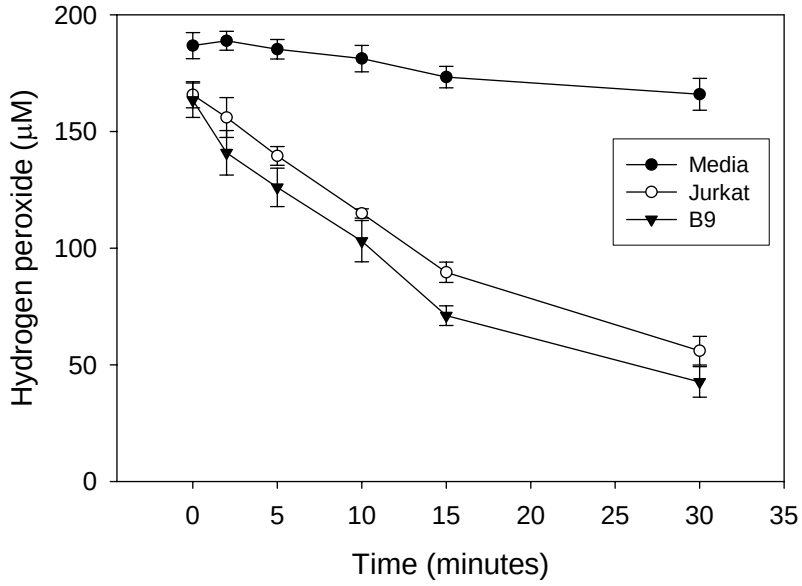


Figure 4.15: Assessing H_2O_2 consumption in Jurkat and B9 cells treated with a bolus of H_2O_2. *Jurkat and B9 cells were cultured in fresh RPMI at a concentration of $1x10^6$ cells/ml prior to the addition of either a 50 μM (A) or a 200 μM (B) bolus of H_2O_2. Aliquots of H_2O_2 treated cell culture or media alone were removed at various time-points and added to FOX reagent. Samples were left to incubate for 40 minutes before being read on a spectrophotometer at 560 nm and related back to a H_2O_2 standard curve. Values represent the mean ± SE of 3 independent experiments.*

4.4 Discussion

The aim of this chapter was to examine potential antioxidant properties of Bcl-2. No changes were observed in any antioxidant enzymes that could explain the resistance of Jurkat cells overexpressing Bcl-2 to H_2O_2-induced apoptosis. The overexpression of Bcl-2 had no affect on the activity of CuZn-SOD, Mn-SOD, catalase, or Gpx. Previous studies have failed to reach a consensus as to whether or not the overexpression of Bcl-2 enhances the activity of these antioxidant enzymes (Table 4.1) (Steinman 1995; Ellerby et al. 1996; Bruce-Keller et al. 1998; Papadopoulos et al. 1998; Amstad et al. 2001; Del Bufalo et al. 2001; Lee et al. 2001; Jang and Surh 2004b). Investigators that have examined Mn-SOD activity in cells overexpressing Bcl-2 found no change when compared to untransfected controls (Bruce-Keller et al. 1998; Lee et al. 2001; Jang and Surh 2004b). Interestingly, overexpression of the Bcl-2 homologue, Bcl-x_L, does not alter the activity of any of these antioxidant enzymes (Bojes et al. 1997). Furthermore, Bcl-2 deficient mice display a marked variability in Gpx activity that is age-dependent (Hochman et al. 2000). These studies underscore the complex nature of any relationship between Bcl-2 expression and antioxidant enzyme activity.

Antioxidant enzyme	Increase	No change
CuZn-SOD	Steinman 1995. Ellerby et al. 1996. Bruce-Keller et al. 1998. Papadopoulos et al. 1998. Lee et al. 2001.	Amstad et al. 2001.
Mn-SOD		Bruce-Keller et al. 1998. Lee et al. 2001. Jang et al. 2004b.
Catalase	Steinman 1995. Ellerby et al. 1996. Bruce-Keller et al. 1998. Lee et al. 2001. Del Buffalo et al. 2001. Jang et al. 2004b.	Ellerby et al. 1996. Amstad et al. 2001. Lee et al. 2001.
Gpx	Bruce-Keller et al. 1998. Papadopoulos et al. 1998. Lee et al. 2001.	Ellerby et al. 1996. Amstad et al. 2001. Lee et al. 2001.

Table 4.1: Summary table displaying the effect of Bcl-2 expression on antioxidant enzyme activities in transfected cells.

The disparity between our study and others showing that Bcl-2 has antioxidant properties may reflect differences in the cell lines used (Hockenbery et al. 1993; Ellerby et al. 1996; Schor and Kagan 1997; Lee et al. 2001; Kowaltowski et al. 2004). Indeed, investigators who have used human lymphoma cell lines have come to a similar conclusion that there are no antioxidant aspects to Bcl-2 function (Lee and Shacter 1997). The cell line-dependence of the "Bcl-2 antioxidant phenomenon" has been emphasised by several studies demonstrating that Bcl-2 overexpression can alter antioxidant enzyme activity and thiol antioxidant capacity in some transfected cell types but not others (Ellerby et al. 1996; Schor et al. 2000; Lee et al. 2001).

Many of the studies reporting that Bcl-2 has antioxidant properties have been performed using only a single Bcl-2 clone (Hockenbery et al. 1993; Mirkovic et al. 1997; Tyurina et al. 1997; Papadopoulos et al. 1998; Rimpler et al. 1999; Amstad et al. 2001; Jang and Surh 2004a). Results attained by comparing a single Bcl-2 clone to the parental cell line need to be interpreted with caution, for several reasons. Firstly, the minor fluctuations seen between the cell lines in the current study highlight the importance of using multiple clones, so that differences observed can be correlated to the level of Bcl-2 expression. Otherwise, minor alterations may mistakenly be interpreted as Bcl-2 dependent changes in the antioxidant capacity of cells. Secondly, the selection procedure used to isolate Bcl-2 clones, which involves exposing cells to high levels of geneticin, may promote the survival of clones with elevated antioxidant status, independent of the transgene involved.

To the best of my knowledge, this was the first time that the expression or activity of Prx and Trx Red was examined in cells overexpressing Bcl-2. Consistent with our earlier observations in this chapter, Bcl-2 overexpression did not modulate the expression or activity of these antioxidant enzymes. The lack of any changes in Prx expression is of special interest given the ability of Prx to participate in apoptotic redox signalling (Rhee et al. 2005a). For instance, overexpression of the cytosolic (Prx-2) or mitochondrial (Prx-3) isoforms of Prx can prevent apoptosis (Zhang et al. 1997; Chang et al. 2004), while reducing the expression of endogenous Prx 3, by RNA interference (siRNA), sensitises cells to apoptotic stimuli (Chang et al. 2004).

The current study has shown that Bcl-2 expression has no discernable effect on either the intracellular thiol concentration or the level of protein thiols. These data suggest that Bcl-2 does not enhance the thiol antioxidant capacity of Jurkat cells. The protein thiol concentrations found in this study (~ 30 nM/mg protein) are in accordance with values determined in previous investigations (Morina et al. 2001; Beer et al. 2004). The data conflict with earlier reports that have found a noticeable increase in the level of protein thiols in cells overexpressing Bcl-2 (Ellerby et al. 1996; Tyurin et al. 1998). However, as described previously, such contrasting results may reflect the cell-line dependence of thiol homeostasis (Schor et al. 2000).

The rate of H_2O_2 clearance was very similar in both the Jurkat and B9 cells, suggesting that Bcl-2 overexpression did not affect H_2O_2 catabolism. Previous studies examining H_2O_2 consumption in Jurkat cells found strikingly similar rates of degradation, confirming that the methodology used to monitor H_2O_2 was sound (Hampton and Orrenius 1997). Our findings are in agreement with those of several groups (Lee and Shacter 1997; Zhao et al. 2000), one of which demonstrated that Bcl-2 expression did not correlate with H_2O_2 decomposition. Together, these results bring into question the hypothesis that Bcl-2 enhances the activity of H_2O_2-decomposing enzymes.

In summary, the results in this chapter have clearly shown that Bcl-2 does not enhance the antioxidant capacity of Jurkat cells. Furthermore, there was no correlation between Bcl-2 expression and the expression or activity of any of the antioxidant enzymes studied. Together, these findings suggest that Jurkat cells overexpressing Bcl-2 are no better at detoxifying oxidative insults than control cells. Therefore, such cells may well exhibit a similar level of oxidative damage as the parental Jurkat cells in response to treatment with H_2O_2. Assessing the effect of Bcl-2 on oxidative damage following exposure to H_2O_2 is the focus of the next chapter.

Chapter 5: Assessing oxidative damage following treatment with H_2O_2 in Bcl-2 transfectants

5.1 Introduction

H_2O_2 exposure can oxidatively damage cellular constituents such as lipids, protein and DNA (Finkel and Holbrook 2000). The oxidation of DNA by H_2O_2 is especially harmful because it promotes mutagenicity and genomic instability (Limoli and Giedzinski 2003; Valko et al. 2004; Zhivotovsky and Kroemer 2004). The overexpression of Bcl-2 has been shown to prevent many forms of oxidative damage (Kowaltowski and Fiskum 2005). Hockenbery and co-workers originally showed, in their groundbreaking study, that Bcl-2 overexpression could block H_2O_2-mediated lipid peroxidation (Hockenbery et al. 1993). Since this landmark paper, several other groups have demonstrated that the overexpression of Bcl-2 could prevent oxidant-mediated lipid peroxidation (Fabisiak et al. 1997; Tyurina et al. 1997; Zhang et al. 1997; Bruce-Keller et al. 1998). Bcl-2 has also been shown to lower protein carbonyl formation in response to oxidative stress (Lee et al. 2001). Furthermore, Bcl-2 deficient mice show increased levels of protein carbonyls, supporting the proposal that Bcl-2 prevents protein oxidation (Hochman et al. 1998; Hochman et al. 2000). Other reports have revealed that the overexpression of Bcl-2 prevents the accumulation of both genomic and mitochondrial DNA damage (Deng et al. 1999; Godley et al. 2002; Mandavilli et al. 2005). However, the contradictory findings of various studies have made it difficult to accept that Bcl-2 universally prevents oxidative damage to all cell constituents (Zhong et al. 1993; Kuo et al. 1999; Seyfried et al. 2003). Indeed, in some circumstances, it seems that Bcl-2 overexpression can exacerbate oxidative damage to both lipids (Seyfried et al. 2003) and DNA (Kuo et al. 1999) in response to oxidative insult.

While it is possible that Bcl-2 itself directly protects against H_2O_2, it is also possible that Bcl-2 expression indirectly affects cellular oxidation. For example, there is increasing evidence that apoptosis leads to increased oxidant production (Zamzami et al. 1995; Um et al. 1996; Cai and Jones 1998), and by impairing apoptosis Bcl-2 appears to be an antioxidant. Alternatively, if apoptotic stimuli, including H_2O_2, cause

oxidative damage, blocking apoptosis in these cells has the potential to result in the accumulation of cells with oxidative damage.

The main objectives of this chapter were:

1) To assess the level of lipid peroxidation occurring in Jurkat cells overexpressing Bcl-2.

2) To determine the extent of protein carbonyls and thiol protein oxidation in Jurkat cells overexpressing Bcl-2.

3) To assess genomic instability in Jurkat cells overexpressing Bcl-2.

5.2 Experimental approach

Jurkat cells and Bcl-2 transfected Jurkat clones (B9 cells) were cultured and challenged with H_2O_2 as described in previous chapters (Section 2.2, 2.3 and 3.2). Lipid peroxidation was assessed by using an oxidant-sensitive lipophilic dye that fluoresces green upon oxidation (Drummen et al. 2002). Previous work in our lab has demonstrated that glyceraldehyde phosphate dehydrogenase (GAPDH) and peroxiredoxin are susceptible to thiol oxidation (Baty et al. 2005). GAPDH thiol oxidation was monitored by using a fluorescent labelling method established in our laboratory (Section 2.18) (Baty et al. 2002). Peroxiredoxin overoxidation was monitored by immunoblotting with a polyclonal antibody specifically raised against the overoxidised form of the protein (Woo et al. 2003b). Protein carbonyls were measured using an enzyme-linked immunosorbent assay (ELISA) developed in our laboratory (Buss et al. 1997). Cell growth was monitored by counting viable cells under a light microscope on a daily basis. Genomic instability was measured by using the cytokinesis-block micronuclei (CBMN) assay (Fenech 2000, 2002). The principle of the assay is to block treated cells at telophase and scan the resulting binucleated cells for nuclear aberrations known as micronuclei (Hogstedt et al. 1981; Fenech 2002).

5.3 Results

5.3.1 Effect of H_2O_2 on lipid peroxidation in Jurkat and B9 cells

Jurkat and B9 cells were loaded with C11-BODIPY$^{581/591}$ for 30 min before exposure to 100 μM H_2O_2 for 1 hour and subsequent assessment of lipid peroxidation (green fluorescing C11-BODIPY$^{581/591}$). Treated cells were visualised by fluorescence microscopy and analysed by flow cytometry. Challenging cells with H_2O_2 led to an increase in green fluorescence for both Jurkat and B9 cells (Figure 5.1). Furthermore, flow cytometry analysis revealed that H_2O_2 exposure caused a similar shift in green fluorescence for both cell lines (Figure 5.2A). Quantification of the flow cytometry data confirmed that the extent of lipid peroxidation was the same in Jurkat and B9 cells (Figure 5.2B).

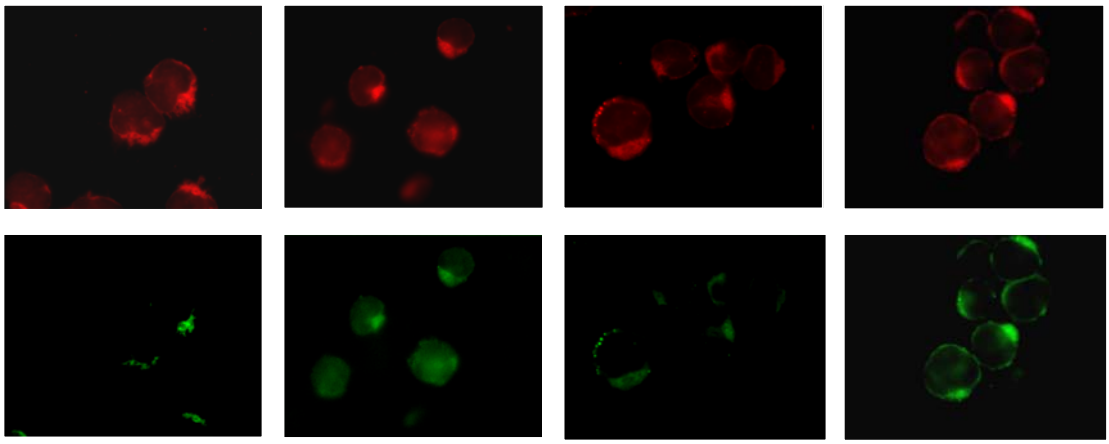


Figure 5.1: Visualisation of lipid peroxidation in Jurkat and B9 cells following H_2O_2 exposure. *Lipid peroxidation was observed by loading cells with the oxidant–sensitive probe C11-BODIPY$^{581/591}$ and assessing changes in fluorescence following exposure to 100 μM H_2O_2. C11-BODIPY$^{581/591}$ fluoresces red in untreated cells (top row), but becomes oxidised fluorescing green (bottom row), following H_2O_2 exposure. Photomicroscopy images are representative of 3 independent experiments.*

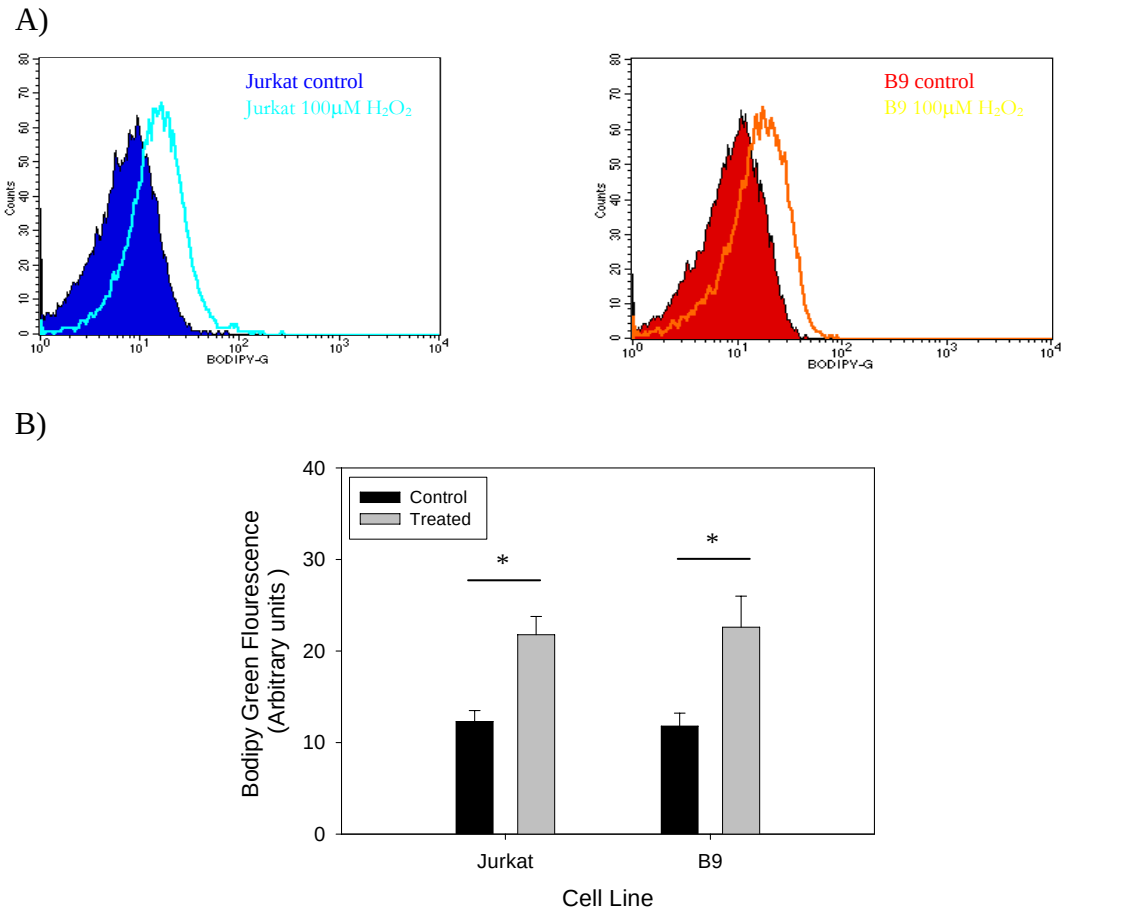


Figure 5.2: Quantifying lipid peroxidation in Jurkat and B9 cells treated with H_2O_2. *Jurkat and B9 cells were loaded with C11-BODIPY$^{581/591}$ and exposed to 100 µM H_2O_2 for 1 hr before being harvested and subjected to flow cytometry analysis for BODIPY-Green fluorescence (A). Values represent the mean ± SE of at least 4 independent experiments (B). * Indicates a significant difference ($p<0.05$) between variables as determined by a One Way Repeated Measures ANOVA with Bonferroni multiple comparison (SigmaStat).*

5.3.2 Measuring GAPDH thiol oxidation in response to H_2O_2

The abundance of GAPDH and the reactivity of its cysteine residues make GAPDH thiol oxidation a good marker of protein oxidation (Little and Obrien 1969; Baty et al. 2005). The dose-dependency of GAPDH thiol oxidation was examined in Jurkat and B9 cells treated with H_2O_2. Since the B9 cells were more resilient than Jurkat cells at higher doses of H_2O_2, one might imagine that the B9 cells would have shown fewer oxidised thiols. Interestingly, this was not the case; thiol oxidation at each H_2O_2 dose was indistinguishable (Figure 5.3A). Densitometry analysis of the GAPDH band intensity confirmed that there was a dose-dependent increase in thiol oxidation that was unaffected by Bcl-2 overexpression (Figure 5.3B).

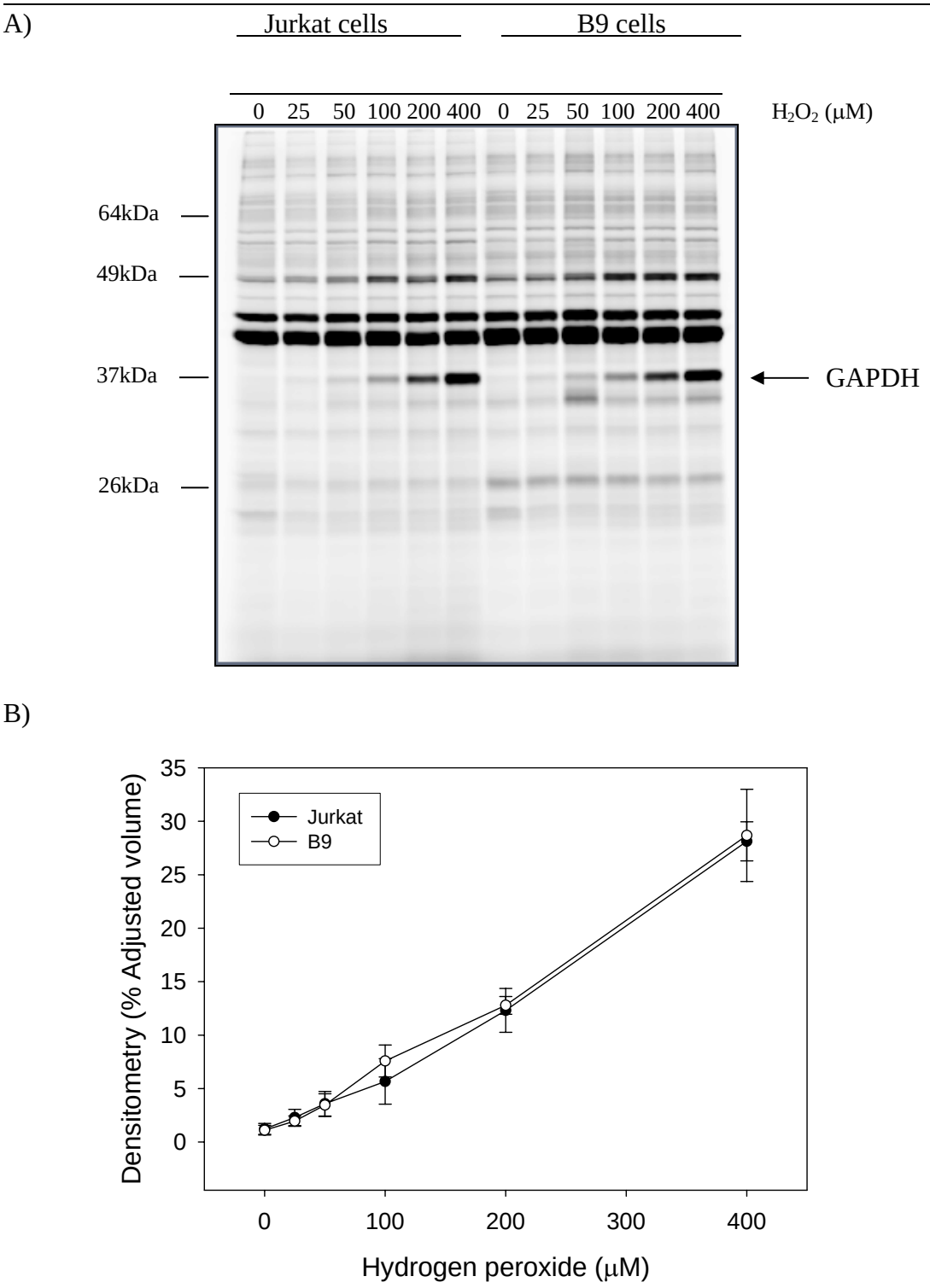


Figure 5.3: GAPDH thiol oxidation in Jurkat and B9 cells exposed to various concentrations of H_2O_2. *GAPDH thiol oxidation was monitored by 5-IAF-labelling in Jurkat and B9 cells exposed to various concentrations of H_2O_2 for 10 min. (A) Illustrative gel of 3 individual experiments displaying oxidised thiol-containing proteins in H_2O_2 treated cells. The oxidised thiols of harvested cell samples were fluorescently labeled, resolved by SDS-PAGE, and imaged using a fluorescent scanner. Gels were subsequently stained with Coommassie blue or silver stain to ensure equal protein loading. B) Densitometry of oxidised thiol-containing GAPDH band intensity. Densitometry was performed using Quantity One (BioRad). Values represent the mean ± SE of at least 3 independent experiments.*

In addition, GAPDH oxidation was assessed in Jurkat and B9 cells treated with a 100 μM bolus of H_2O_2 over a one-hour time period (Figure 5.4). The Jurkat and the B9 cells displayed an approximately linear increase in GAPDH thiol oxidation over time. As predicted, GAPDH thiol oxidation was rapid and could be detected directly after the oxidative insult. Densitometry analysis of the oxidised thiol GAPDH band revealed that the rate of GAPDH thiol oxidation was comparable in both cell lines (Figure 5.5).

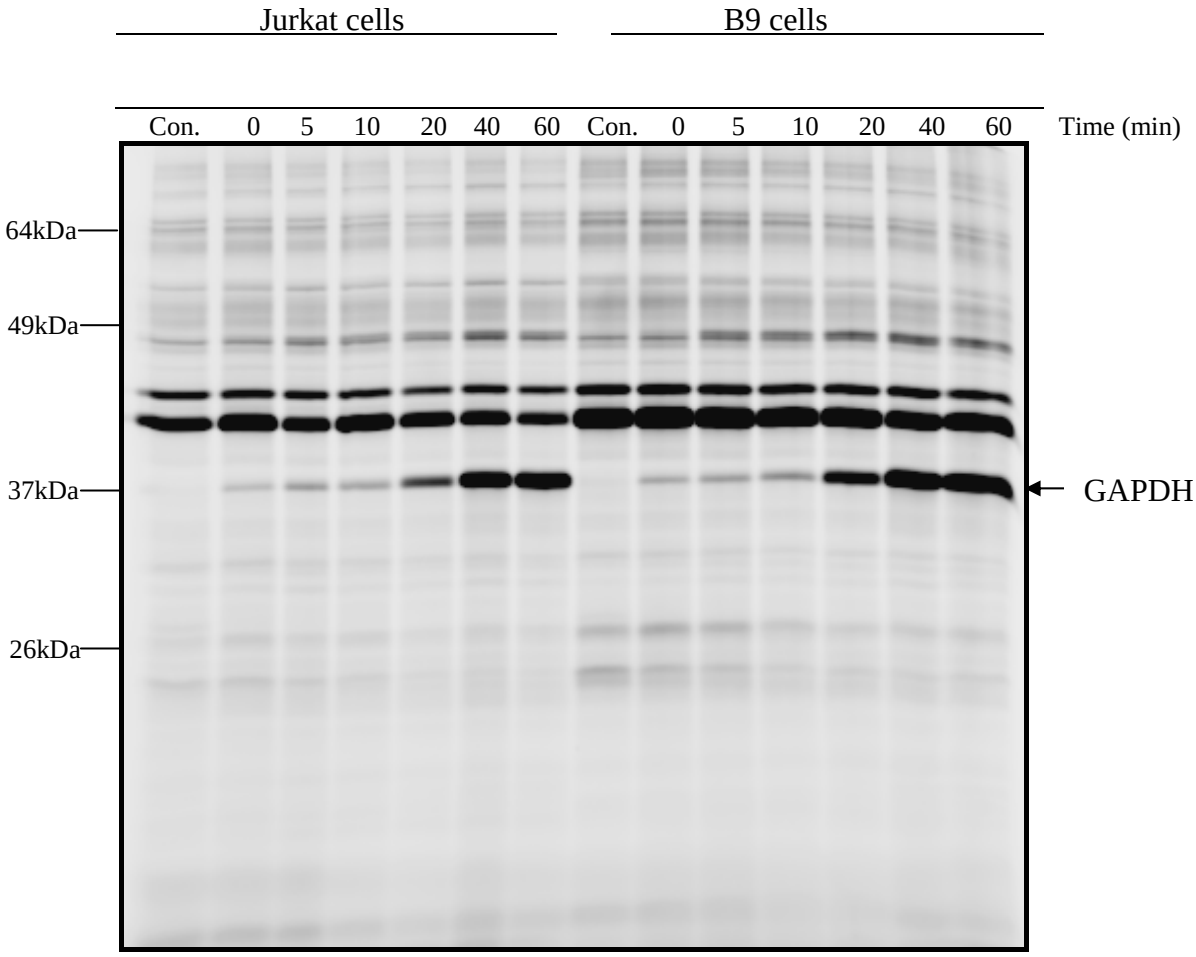


Figure 5.4: GAPDH thiol oxidation in Jurkat and B9 cells treated with H_2O_2 over time. *GAPDH thiol oxidation was monitored over time in Jurkat and B9 cells treated with 100 μM H_2O_2 by 5-IAF-labelling. Illustrative gel of 3 independent experiments displaying oxidised thiol-containing proteins in H_2O_2 treated cells. The oxidised thiols of harvested cell samples were fluorescently labeled, resolved by SDS-PAGE, and imaged using a fluorescent scanner. Gels were subsequently stained with Coommassie blue or silver stain to ensure equal protein loading.*

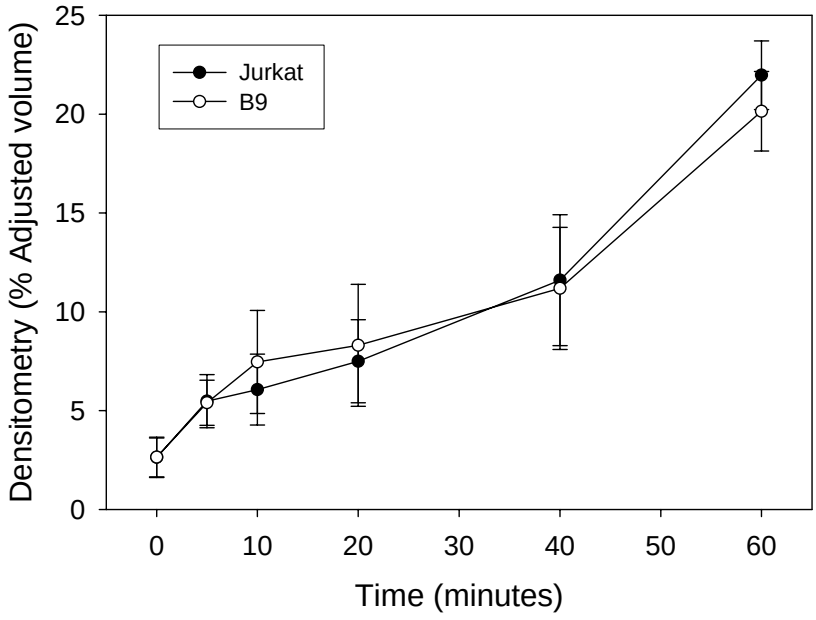


Figure 5.5: Densitometry analysis of GAPDH thiol oxidation in Jurkat and B9 cells treated with H_2O_2 over time. *Jurkat and B9 cells were treated with 100 μM H_2O_2 and oxidised thiols were labeled with 5-IAF. Densitometry was performed using Quantity One (BioRad). Values represent the mean ± SE of at least 3 independent experiments*

5.3.3 Monitoring peroxiredoxin overoxidation in response to H_2O_2

The cysteine residue in the active site of the peroxiredoxins can readily become overoxidised in response to H_2O_2 exposure (Rabilloud et al. 2002; Georgiou and Masip 2003), leading to the formation of the sulfinic acid (Cys-SO_2H), which can be detected immunologically (Woo et al. 2003b). Jurkat and B9 cells were treated with 100 μM H_2O_2 and samples were collected periodically over time in a lysis buffer containing 10 mM NEM. The presence of NEM in the lysis buffer freezes the redox-state of the cell and ensures that no thiol oxidation or reduction takes place (Gitler et al. 1994). Sample lysates were analysed by SDS-PAGE in non-reducing conditions and immunoblotted against the overoxidised (Cys-SO_2H) form of the peroxiredoxins. The immunoblots revealed that the overoxidised form of the peroxiredoxins formed immediately after H_2O_2 exposure in both cell lines (Figure 5.6A). Interestingly, the amount of overoxidation did not decrease during the first hour of treatment. These data suggest that the overoxidised form of the peroxiredoxins remains, even when the level of oxidant has dissipated (section 4.3).

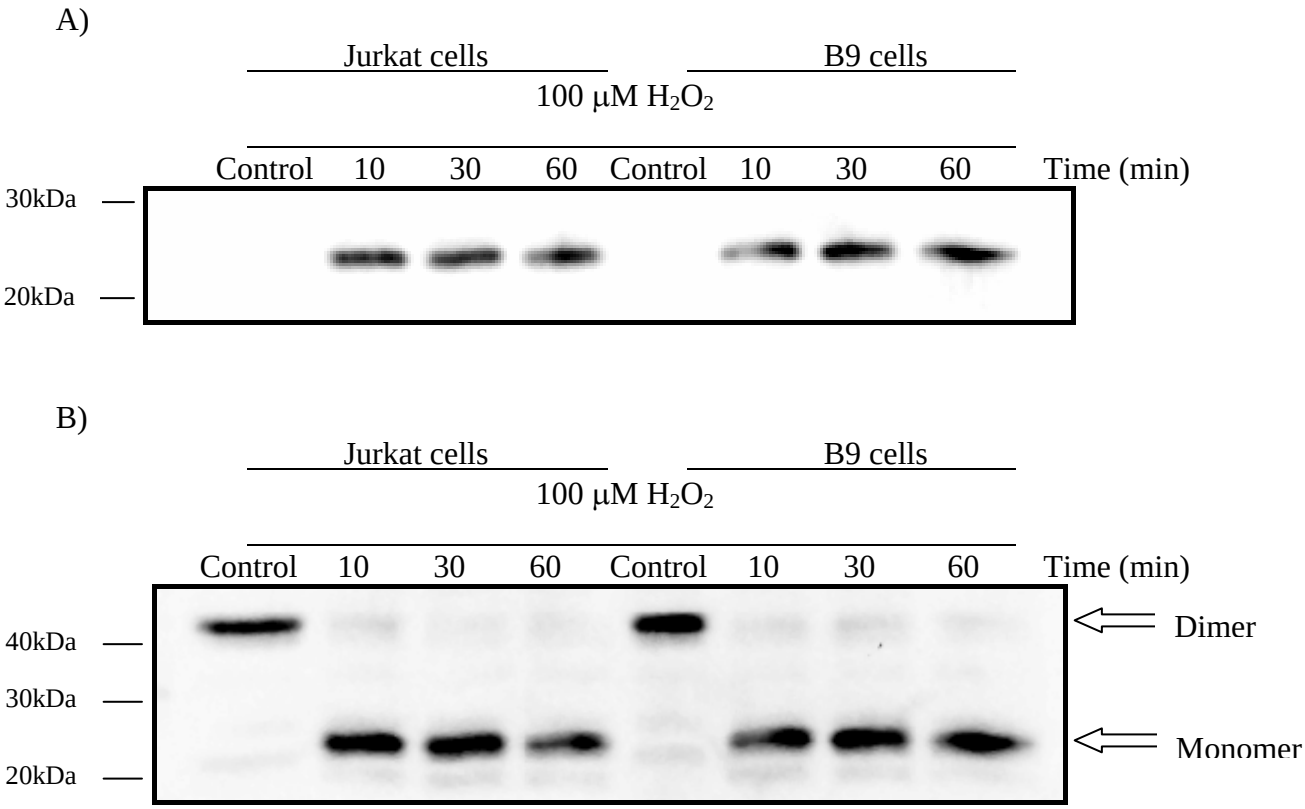


Figure 5.6: Detection of overoxidised peroxiredoxins in Jurkats and B9s treated with H_2O_2. *Illustrative Western blots of 3 independent experiments against Prx-SO_3 (A) and Prx2 (B) in H_2O_2 challenged Jurkats and B9s. Jurkat and B9 cells treated with 100 μM H_2O_2 were harvested periodically over time. Harvested cells were lysed, and mixed with non-reducing sample buffer. Samples were analysed by SDS-PAGE, blotted onto PVDF membrane and immunoblotted for the relevant antibody.*

Stripping and reprobing the membrane for peroxiredoxin 2 confirmed that the differences seen between controls and treated cell lysates were not an artifact of protein loading (Figure 5.6B). Peroxiredoxin 2 forms a disulfide-linked dimer in healthy cells during its catalytic cycle (Rhee et al. 2005). Interestingly, when control lysates were resolved in non-reducing conditions in the absence of NEM, only the dimer was present, suggesting that peroxiredoxin 2 spontaneously dimerises upon lysis. This phenomenon has been observed previously in the laboratory (Felicia Low, personal communication). When peroxiredoxin 2 is overoxidised it is unable to form dimers. The fact that there was no dimer present in the treated cells suggests that the entire pool of peroxiredoxin 2 becomes overoxidised in Jurkat and B9 cells treated with 100 μM H_2O_2.

5.3.4 Effect of H_2O_2 on protein carbonyl formation in Jurkat and B9 cells

Another approach used to assess oxidative stress in response to 100 μM H_2O_2 was to determine the extent of protein carbonyl formation. The protein carbonyl data demonstrated that B9 cells formed fewer protein carbonyls over time in response to H_2O_2 than Jurkat cells (Figure 5.7). The level of protein carbonyls formed in B9 cells reached a maximum 1 hour after H_2O_2 treatment (0.144 nM/mg $\pm$ 0.031). In contrast, the Jurkat cells continued to form protein carbonyls over time, reaching a maximum five hours after treatment (0.440 nM/mg $\pm$ 0.05).

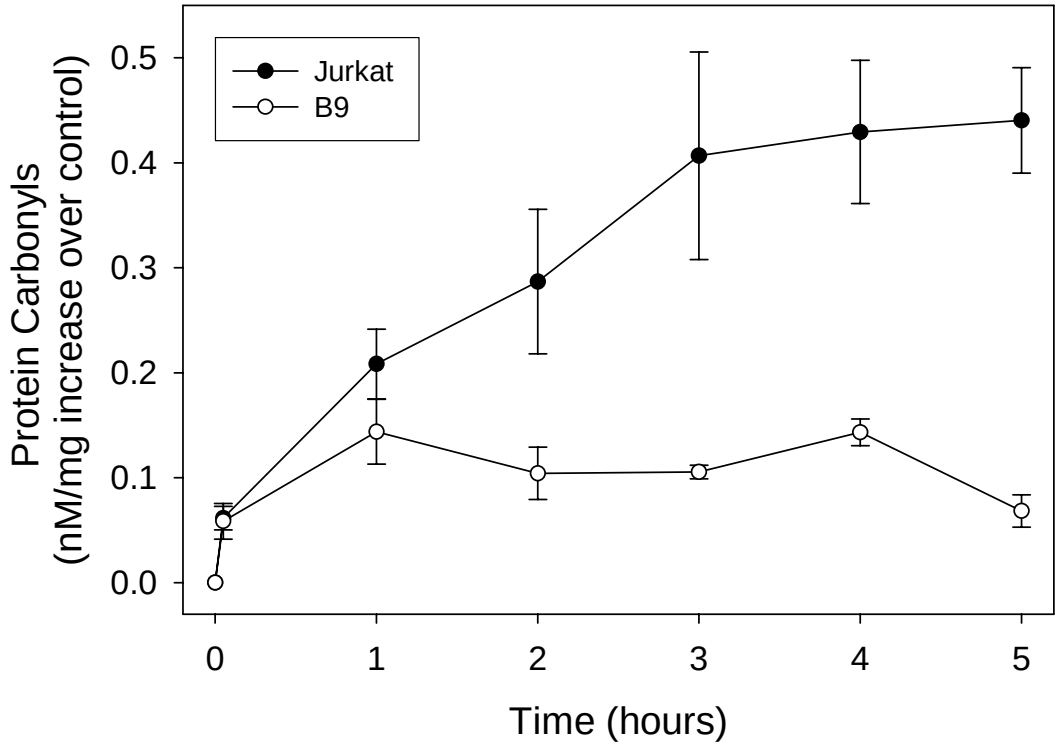


Figure 5.7: Protein carbonyl formation in Jurkats and B9s treated with H_2O_2. *Jurkat and B9 cells treated with 100 μM H_2O_2 were harvested periodically over time and kept at – 80°C. Protein carbonyls were labeled with DNP and adsorbed to a high-bind microtitre plate. Protein carbonyls were detected by ELISA using a streptavidin-conjugated HRP detection system. Values represent the mean $\pm$ SE of at least 3 independent experiments.*

5.3.5 Effect of H_2O_2 on growth in Jurkat and B9 cells

The adverse affects of oxidative stress have been shown to affect the proliferative capacity of cells (Finkel and Holbrook 2000). Having demonstrated that Jurkat cells overexpressing Bcl-2 fail to undergo apoptosis in response to H_2O_2 exposure (Chapter 3), yet show signs of oxidative stress, it was of interest to investigate whether the damaged cells continued to proliferate. To address this, Jurkat and B9 cells were treated with 100 μM H_2O_2 and their long-term growth was assessed (Figure 5.8). The data demonstrated that when Jurkat cells were treated with H_2O_2, they progressively died, reaching the lowest concentration of viable cells after 3 days ($0.11x10^6$ cells $\pm$ 0.08). After 8 days, those surviving Jurkat cells had shown signs of proliferation. In contrast, when the B9 cells were treated with H_2O_2, they went into a temporary growth arrest for a 2-3 day period, without significant cell loss (consistent with Figure 3.8). The growth-arrested cells were viable and metabolically active, requiring daily media change. At approximately 3-4 days, the cells began to proliferate again, albeit at a reduced rate to untreated cells.

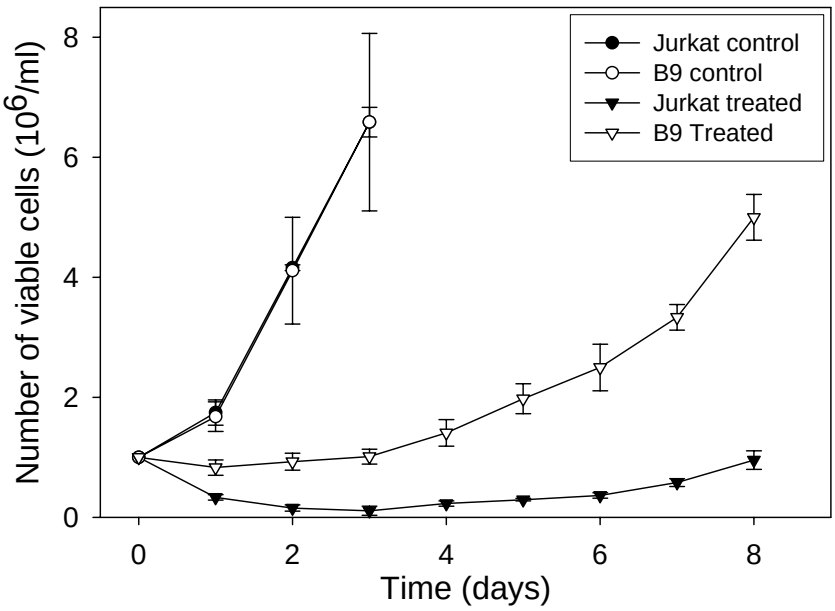


Figure 5.8: Monitoring growth of Jurkat and B9 cells treated with H_2O_2 over time. *The growth of treated Jurkat and B9 cultures was monitored daily by counting cells with a hemocytometer using the trypan blue exclusion assay. The culture medium was replaced daily to avoid overgrowth. Values represent the mean $\pm$ SE of at least 4 independent experiments.*

5.3.6 Effect of H_2O_2 on genomic instability in Jurkat and B9 cells

Having demonstrated that Jurkat cells overexpressing Bcl-2 survive and continue to grow after H_2O_2 exposure, it was of interest to determine whether the surviving cells showed signs of genomic instability. The cytokinesis-block micronuclei (CBMN) assay is a common method used to detect chromosome breakages in lymphocytes exposed to genotoxic stress (Fenech et al. 1999; Fenech 2000, 2002). Micronuclei, also known as Howell-Jolly bodies to haematologists (Hutchison and Fergusonsmith 1959), are chromosomal fragments resulting from chromosome breakages that cannot migrate to the spindle poles during mitosis (Hogstedt et al. 1981; Fenech 2002). Jurkat and B9 cells were exposed to H_2O_2 for four hours before being treated with the microfilament assembly inhibitor chytochalasin B, which blocks replicating mammalian cells in telophase, for a further 24 hours. The binucleated cells that resulted from chytochalasin B treatment were examined microscopically to detect chromosomal breakages (micronuclei formation) caused by H_2O_2 exposure (Figure 5.9).

The CBMN data revealed that Jurkat cells exhibited a very low basal level of micronucleated cells in control conditions; however, the number substantially increased after treatment with 50 μM H_2O_2 (Figure 5.10). Jurkat cells exposed to 100 μM H_2O_2 became apoptotic or necrotic, making micronucleated cell counts unfeasible. Strikingly, B9 cells showed a dramatic increase in the amount of micronucleated cells in both basal conditions and following treatment with H_2O_2. B9 cells exposed to 100 μM H_2O_2 expressed an extremely high level of micronucleated cells (~3.5%).

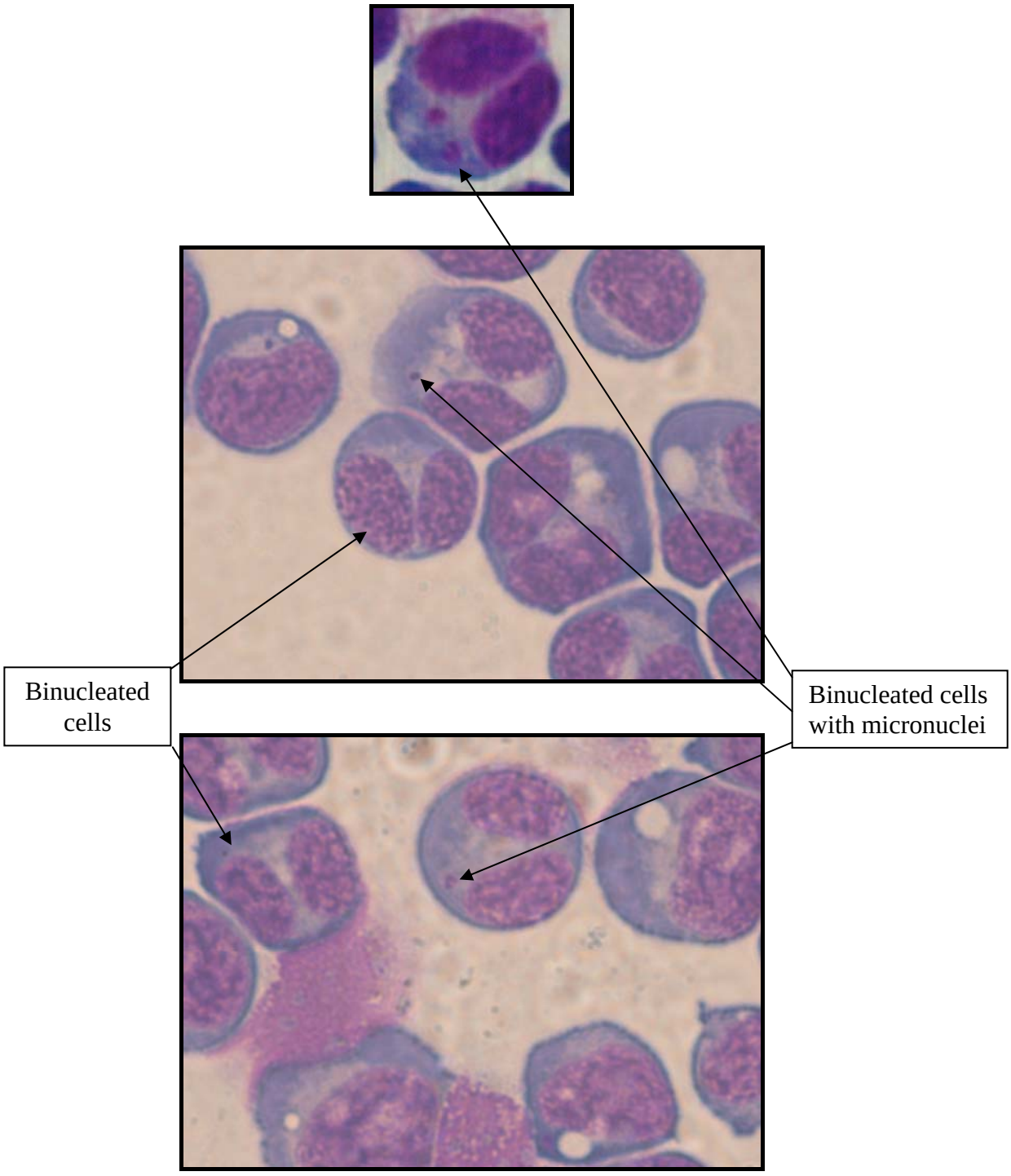


Figure 5.9: Illustrative photographs of cells used for CBMN scoring. *Photos are representative of at least 3 independent experiments. The photos show examples of binucleated cells (dividing cells) and binucleated cells with micronuclei (dividing cells harboring chromosomal breakages).*

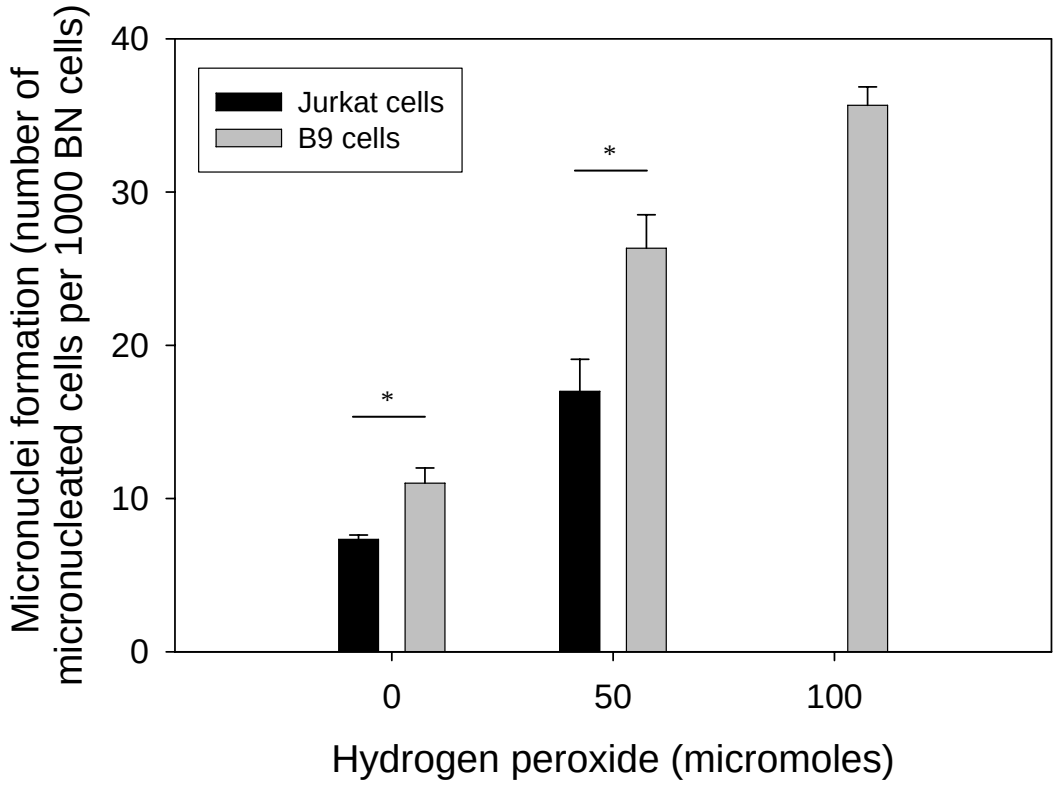


Figure 5.10: Micronuclei formation in Jurkat and B9 cells treated with H_2O_2. *Histogram of MN formation in Jurkats and B9 cells treated with 50 μM and 100 μM H_2O_2. Treated cells were harvested and transferred to a glass slide by cytocentrifugation. MN were scored using criteria as previously described (Fenech et al. 2003). Values represent the mean $\pm$ SE of at least 3 independent experiments. * Indicates a significant difference ($p<0.05$) between variables as determined by a One Way Repeated Measures ANOVA with Bonferroni multiple comparison (SigmaStat).*

5.4 Discussion

The results from this study clearly show that Jurkat cells overexpressing Bcl-2 suffer the same extent of lipid peroxidation in response to H_2O_2 as the parental cell line. These results are in accordance with a study by Lee and coworkers (Lee et al. 2001) and suggest that Bcl-2 overexpression does not enhance lipid antioxidant or repair systems in Jurkat cells. The use of the C-11 BODIPY probe to detect lipid peroxidation has been validated in an extensive study by Drummen et al. (Drummen et al. 2002). Interestingly, several of the conflicting reports that suggest Bcl-2 overexpression can prevent lipid peroxidation, including the original study by Hockenbery and colleagues, detected differences at later time-points (Hockenbery et

al. 1993; Tyurina et al. 1997). Differences seen at such time-points need to be interpreted with caution, as the induction of apoptosis can generate oxidants (Zamzami et al. 1995; Polyak et al. 1997; Chandra et al. 2000) that may obscure real differences seen due to antioxidant properties of Bcl-2. For example, Cai and Jones (Cai and Jones 1998; Cai et al. 1998) have shown that cytochrome c, released from the mitochondria of apoptotic cells, can cause an increase in the production of superoxide. In the same study, Bcl-2 inhibited superoxide production by maintaining cytochrome c in the mitochondria. This work highlights that Bcl-2 can appear to possess antioxidant properties by virtue of regulating the apoptotic pathway. In addition to ROS production, apoptosis is often accompanied by a depletion of the thiol reductant glutathione via oxidation (Macho et al. 1997) or efflux (van den Dobbelsteen et al. 1996; Pullar and Hampton 2002). The loss of glutathione during the early stages of apoptosis can leave the cell susceptible to oxidative damage (Hall 1999).

Protein carbonyl formation was also examined in H_2O_2 treated Jurkat cells overexpressing Bcl-2. The elevated level of oxidants produced during apoptosis may explain why the concentration of protein carbonyls continued to increase in Jurkat cells at later time-points. At these time-points (3-5 hr), Jurkat cells treated with 100 μM H_2O_2 were beginning to exhibit apoptotic morphology. Protein carbonyl formation was comparable in both cell lines at early time-points, suggesting that the initial oxidant exposure caused a similar level of protein oxidation in Jurkat cells overexpressing Bcl-2.

An alternative explanation is that cells overexpressing Bcl-2 may dispose of protein carbonyls more efficiently. Previous studies have shown that protein carbonyls formed by H_2O_2 exposure are effectively removed by the proteasome (Sitte et al. 1998). Interestingly, Lee et al. (Lee et al. 2001) have recently demonstrated that cells overexpressing Bcl-2 have a higher proteasomal activity. Therefore, it is possible that the low level of protein carbonyls seen in cells overexpressing Bcl-2 is a reflection of the enhanced proteasomal activity, as opposed to any antioxidant properties of Bcl-2. This would be of interest for further investigation.

The overexpression of Bcl-2 had no effect on the level of oxidised GAPDH or peroxiredoxin thiols following exposure to H_2O_2. The pattern of thiol oxidation seen in the IAF-labeled gels was similar to that seen by Baty et al. (Baty et al. 2005), with the prominent GAPDH band becoming oxidised at low levels of H_2O_2. GAPDH thiols were oxidised in a time and dose-dependent manner in both cell lines. To the best of my knowledge this was the first time peroxiredoxin overoxidation has been examined in cells overexpressing Bcl-2. In support of previous studies, our results clearly showed that peroxiredoxin 2 was completely overoxidised in response to 100 μM H_2O_2 and is not immediately regenerated within the first hour of exposure (Rabilloud et al. 2002; Chevallet et al. 2003; Woo et al. 2003a; Woo et al. 2003b).

Jurkat cells overexpressing Bcl-2 showed very distinct differences in cell growth after H_2O_2 exposure when compared with control cells. Jurkat cells exposed to 100 μM H_2O_2 progressively died; however, Jurkat cells overexpressing Bcl-2 became temporarily growth-arrested before continuing to proliferate. Previous studies have demonstrated that the dose of H_2O_2 is critical in the cells decision to either undergo growth arrest or apoptosis (Davies 1999). The overexpression of Bcl-2 appears to bias the cells decision, blocking apoptosis and therefore promoting alternative stress responses, such as temporary growth arrest. It is possible that in the growth-arrested state, cells overexpressing Bcl-2 repair oxidative damage and they re-enter the cell cycle.

This study demonstrated, for the first time, that the overexpression of Bcl-2 enhances the proportion of micronucleated cells seen in Jurkat cells following H_2O_2 exposure. The results attained by exposing Jurkat cells to H_2O_2 are very similar to those reported previously by Fenech et al. (Fenech et al. 1999). The fact that Bcl-2 increased the proportion of micronucleated cells is consistent with a previous study demonstrating that cells overexpressing Bcl-2 have a higher frequency of micronuclei formation following X-irradiation (Taga et al. 2000). These findings are also in accordance with the results of several other groups investigating the effect of Bcl-2 on oxidant-mediated mutagenesis (Steinman 1995; Cherbonnel-Lasserre et al. 1996a, 1996b; Cherbonnel-Lasserre and Dosanjh 1997; Kuo et al. 1999; Saintigny et al. 2001). Such studies have consistently found that Bcl-2 overexpression enhances DNA and chromosomal damage, promoting mutagenesis in cells exposed to an oxidative insult.

Our findings are in line with a number of elegant *in vivo* studies showing that transgenic mice overexpressing Bcl-2 are prone to cancer and chromosomal instability (Reed et al. 1988; Strasser et al. 1990; McDonnell and Korsmeyer 1991; Gibbons et al. 1999; Letai et al. 2004; Oltersdorf et al. 2005). The data reported herein demonstrate that by overriding apoptosis, Bcl-2 perturbs the physiological surveillance of genomic instability and renders cells more susceptible to tumourigenesis.

Bcl-2 not only enhances mutagenicity by suppressing apoptosis, it may also promote mutagenesis by elevating basal ROS production (Steinman 1995; Kowaltowski et al. 2004) and impairing several DNA repair pathways (Liu et al. 1997; Zhan et al. 1999; Gu et al. 2001; Saintigny et al. 2001; La Thangue 2005; Youn et al. 2005). The overexpression of Bcl-2 suppresses the nucleotide excision repair pathway by downregulating the expression of key DNA glycosylase enzymes hOGG1 and hMYH (Liu et al. 1997; Gu et al. 2001). These enzymes are responsible for removing chemically altered DNA bases such as 8-oxo-dG. In addition, Bcl-2 overexpression inhibits the RAD51-dependent homologous recombination repair pathway (Zhan et al. 1999; Saintigny et al. 2001). This pathway is fundamental in repairing DNA double strand breaks that might result from oxidant exposure. Overexpression of the Bcl-2 mutant ^{G145A}Bcl-2, which is defective in binding to BH3 proteins (Huang et al. 1997), inhibits the RAD51 recombination pathway (Saintigny et al. 2001), demonstrating that the anti-apoptotic properties of Bcl-2 can be genetically separated from its inhibitory effect on the RAD51 recombination pathway. Recent studies have shown that Bcl-2 overexpression also suppresses hMSH2, a DNA mismatch repair enzyme involved in repairing lesions in DNA (La Thangue 2005; Youn et al. 2005). Collectively, these studies reveal that Bcl-2 overexpression can suppress DNA repair pathways resulting in a mutator phenotype. It is likely that the Bcl-2-dependent decrease in DNA repair activity is a contributing factor in the oxidant-mediated mutagenesis of cells overexpressing Bcl-2.

In summary, the results of this chapter have clearly demonstrated that Jurkat cells overexpressing Bcl-2 exhibit the same level of oxidative damage to lipids, protein and DNA as the parental Jurkat cells in response to H_2O_2 exposure. Furthermore, H_2O_2-treated Jurkat cells overexpressing Bcl-2 continued to proliferate, despite damage to

cellular constituents. The inability of Jurkat cells overexpressing Bcl-2 to undergo apoptosis meant that they were more susceptible to genomic instability as a result of H_2O_2 exposure.

Chapter 6: Summary and Future Directions

6.1. Summary of Findings

The oxidant H_2O_2 can participate in a variety of damaging reactions with cellular constituents (Finkel and Holbrook 2000) that can activate apoptosis and necrosis (Chandra et al. 2000; Zong and Thompson 2006). The apoptotic pathway of cell death can be blocked by overexpression of the oncoprotein Bcl-2 (Green and Kroemer 2004). Some investigators believe that Bcl-2 possesses antioxidant properties that protect cells from apoptosis in response to oxidants (Kowaltowski and Fiskum 2005). In this study, we investigated the cytotoxic and damaging properties of H_2O_2 in Jurkat cells overexpressing Bcl-2. In particular, we investigated whether the overexpression of Bcl-2 provides any protection against H_2O_2.

We have shown that Jurkat cells exposed to H_2O_2 undergo apoptosis or necrosis depending on the dosage; intermediate doses favour apoptosis, while higher doses promote necrosis. The overexpression of Bcl-2 prevented cell death at the apoptotic doses, but not the necrotic doses of H_2O_2. However, inhibiting apoptosis with the pan-caspase inhibitor z-VAD.fmk could not provide the same level of resistance against H_2O_2-induced cell death as Bcl-2 overexpression.

These studies spurred investigations into whether Bcl-2 acted in an antioxidant capacity to prevent H_2O_2-induced cell death. The antioxidant systems of Jurkat cells overexpressing Bcl-2 were examined in detail. A number of Jurkat clones overexpressing Bcl-2 were used so that any differences seen could be correlated to the level of Bcl-2 expression. The intention was to build on the substantial body of literature suggesting that Bcl-2 enhances the antioxidant capacity of the cell (Hockenbery et al. 1993; Ellerby et al. 1996; Schor and Kagan 1997; Lee et al. 2001; Kowaltowski et al. 2004). There were no significant differences; however, among the Bcl-2 transfectants in any of the antioxidant enzymes. Together, this data suggested

that Jurkat cells overexpressing Bcl-2 decomposed oxidants such as H_2O_2 no better than the parental Jurkat cells.

Consistent with this observation, Jurkat cells overexpressing Bcl-2 exhibited the same level of oxidative damage to cellular constituents in response to H_2O_2 exposure as the parental Jurkat cells. Interestingly, damaged Jurkat cells overexpressing Bcl-2 that failed to undergo apoptosis continued to proliferate after a period of temporary growth arrest. We also found that Jurkat cells overexpressing Bcl-2 were more susceptible to genomic instability as a result of H_2O_2 exposure.

6.2. Future directions

Although our studies have systematically demonstrated that Bcl-2 does not enhance the antioxidant capacity of Jurkat cells, it may behave in a more subtle manner, altering the redox environment in such a way as to promote cell survival. There are numerous examples in the literature of signaling proteins that are regulated by slight changes in cellular redox (Cross and Templeton 2004). Many of these proteins possess reactive cysteine residues that are sensitive to oxidation (Forman et al. 2004). It would be of interest to examine whether Bcl-2 overexpression predisposed thiol redox sensors to remain in the reduced form. Such alterations would not be detectable in the global assays performed thus far; however, they may be detected using the oxidised thiol proteomics methodology established in our lab by Baty et al. (Baty et al. 2002, 2005). This method enables the identification of low abundance thiol proteins that are sensitive to oxidation.

The fact that Bcl-2 overexpression enhanced the mutagenicity of H_2O_2 in Jurkat cells is of special interest and warrants further investigation. It would be of interest to examine whether the Jurkat cells overexpressing Bcl-2 treated with H_2O_2 exhibit persistant genomic instability. For instance, it would be interesting to see if B9 cells exposed to H_2O_2 retain a higher proportion of micronucleated cells after longer time periods, greater than a week. Furthermore, it may be constructive to investigate the mutagenic effect of Bcl-2 overexpression in a primary cell line exposed to H_2O_2. Such studies would be amenable to comparative global hybridization (CGH), which might

enable the identification of common chromosomal abnormalities that occur in response to H_2O_2 exposure. Based on the findings, it seems likely that the overexpression of Bcl-2 will render cells more susceptible to mutagenesis imposed by other oxidants. Indeed, Kuo et al. (Kuo et al. 1999) have demonstrated that Bcl-2 overexpression enhances mutagenesis imposed by benzene metabolites, which are known to generate ROS. It may also be of interest to investigate the potential role that suppressed DNA repair (Liu et al. 1997; Zhan et al. 1999; Saintigny et al. 2001; Youn et al. 2005) plays in enhancing the mutagenesis of Jurkat cells overexpressing Bcl-2.

In conclusion, the results presented herein provide useful insights into the effect that Bcl-2 overexpression has in protecting Jurkat cells from the cytotoxic action of H_2O_2. The results attained in this thesis, showing the cytoprotective effect of Bcl-2 in Jurkat cells exposed to H_2O_2, provide a platform for exciting research in the future.

Chapter 7: References

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